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AN EXPERIMENTAL STUDY OF THE LYMPHOCYTIC RESPONSE IN THYROTOXICOSIS*

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POSITIVE evidence for the existence of a thyroid-lymphoid relationship is indicated by numerous clinical and pathologic observations (Plummer,[1] Falta,[2] and others) and by numerous experimental studies (Hoskins[3]; Kahn[4]; Gudernatsch[5]; Marine, Manley, and Baumann[6]; and Adams and Shevket[7]; and others), but positive evidence showing whether the lymphatic hyperplasia and consequent lymphocytosis is or is not functionally related to the hyperthyrosis is lacking; although, as Rowntree[8] points out, thymic hyperplasia in exophthalmic goiter has been considered by some as a compensatory hypertrophy, and Gudernatsch concluded that the thymus, spleen, and perhaps other lymphoid tissues as well, constitute glands with a positive internal secretion.

The present study was initiated by the constant observation by one of us (Turley) through thirty years of pathologic investigation that (1) hyperthyrosis is accompanied by a lymphocytosis; (2) the phenomenon of lymphocytic infiltration of the thyroid gland in thyrotoxicosis is interpretable in the light of the law of phagocytic infiltration, viz., that phagocytes infiltrate tissues at the site of pathologic processes; (3) all experimental work so far reported could be interpreted on a functional basis; and (4) the relation, whatever its nature, involved the lymphocytes as cells more than it did lymphatic tissues as organs. In view of these indications it seemed desirable that an attempt be made to determine whether the generalized lymphoid hyperplasia and the lymphocytes in thyrotoxic lymphocytoses are performing a function related to the thyrotoxicosis.

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METHODS

The experimental animals used were guinea pigs weighing between 300 and 500 Gm. each. The animals were divided into three groups. In group I each animal was fed a balanced diet to which was added either 1 or 4 grains of thyroid substance daily. In group II each animal was fed daily a normal diet plus 1.5 Gm. of aleuronat. In group III each animal received daily 1 grain of thyroid and 1.5 Gm. of aleuronat plus a normal diet. Four animals were used in group I, 6 in group II, and 8 in group III.

Total and differential blood counts, hemoglobin estimates, and weighings were made daily on each animal. As nearly as possible, blood was taken from each animal at the same hour every day. To avoid a generalized traumatic reaction in the ear and its possible effects on the blood picture, a single ear vein was severed in the right and left ears on alternate days. When deaths occurred, autopsies were done and tissues of all pertinent organs were taken for microscopic study.

In a preliminary series of studies the animals of groups II and III contracted pneumonia during the course of experimentation. This complicated our results and made it difficult, if not impossible, to distinguish between the immediate effects of the drugs administered and the effects of the pneumonic condition. Because of this complication, and because of the positive indications of these experiments, a second series of groups II and III experiments were performed, during which our animals were maintained free of intercurrent infection. In the following discussion of these studies, all remarks concerning groups II and III have reference to these second series unless definitely stated to pertain to the first and preliminary groups II and III experiments.

RESULTS

Group I: The average survival time of these animals was three days. Of this group animals 1 and 2, receiving 1 grain of thyroid daily, lived 3.2 days; and animals 3 and 4, receiving 4 grains daily, lived only two days (Fig. 1*A*).

All the animals lost weight rapidly. Those receiving 1 grain of thyroid lost 29.5 Gm. daily, and those receiving 4 grains lost 72.5 Gm. daily (Figs. 2, 3*A*).

Erythrocyte counts varied individually and according to the amount of thyroid substance administered. The number of red blood cells increased in those animals receiving 1 grain of thyroid substance and decreased in those receiving 4 grains (Fig. 2).

Four prominent alterations in the leucocytic formula occurred. (1) In all but one animal a persistent leucopenia developed. (2) In all animals lymphocytes were dominant, both absolutely and relatively, during experimentation. (3) All but one animal showed an absolute but variable increase in the number of lymphocytes at the end of the first experimental day, and on all subsequent days an irregular but absolute decrease in lymphocytes occurred. (4) In every case the number of neutrophiles decreased during experimentation (Fig. 2). Significant alterations in the number of eosinophiles, basophiles, and monocytes did not occur.

Group II: No deaths occurred during the course of experimentation which lasted thirty-two days (Fig. 1). Animal 4 was sacrificed for tissue and autopsy on the fifty-first day, and the others on the 120th day. The autopsy material appeared negative.

Except for an occasional variation, all the animals in this group gained weight during the course of experimentation. Animal 2 developed a temporary infection and was disregarded in our interpretations. Animals 1, 3, and 4, considered collectively, gained weight at the rate of 1.3 Gm. daily (Figs. 3*A* and 5). Weight changes in these animals occurred in diphasic cycles; that is, for a period of one to three days weight increases of 3 to 10 Gm. daily occurred, and then for a period of about the same duration little or no weight would be gained, or a little might be lost. Such diphasic cycles are repeated (Figs. 5, 6).

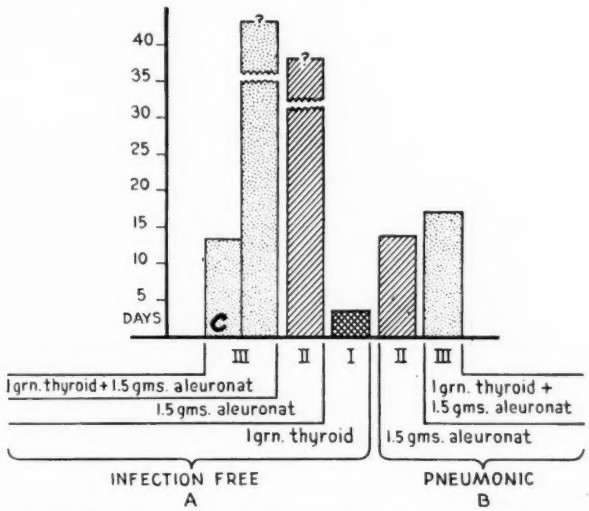

Fig. 1.—A comparative chart showing the survival time in days of the animals of experimental groups I, II, and III. *A*, second series of experiments which were free of intercurrent infection; *B*, preliminary experiments in which pneumonia intervened; *C*, animal 3, the only group III animal to die of this treatment.

Alterations in the peripheral blood were pronounced. The number of red blood cells varied characteristically from day to day and with a moderate increase (Figs. 5, 6). Although the total number of white blood cells showed daily wide variations, the average daily count for animals 1, 3, and 4 during thirty-one experimental days was only 6 per cent above normal (Figs. 5, 6). Changes in the leucocytic formula were the most pronounced and were characterized by an absolute and persistent increase in the number of lymphocytes and by an absolute decrease in neutrophiles (Figs. 4, 5, 6). There were no changes in the number of other blood cell types from normal.

The developmental sequence of these alterations in the leucocytic formula can be easily discerned from the graphs, but it is desirable to point out, first, that there is an immediate rise in the number of lymphocytes, and that by the sixth experimental day the absolute number of lymphocytes may be from one and five-tenths to three times larger than the normal number (Figs. 5, 6); and second, that marked leucocytoses occur rhythmically in cycles which, with a fair degree

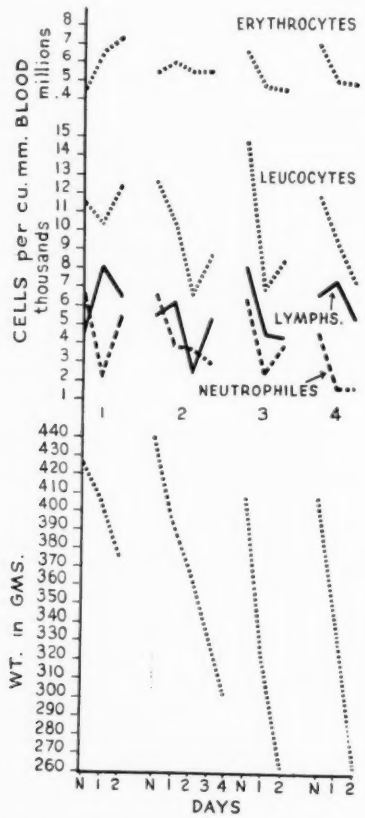

Fig. 2.—A graph of the daily alterations in weight and in the blood picture of the group I animals. Animals 1 and 2 were fed 1 grain of thyroid daily; animals 3 and 4 were fed 4 grains of thyroid daily.

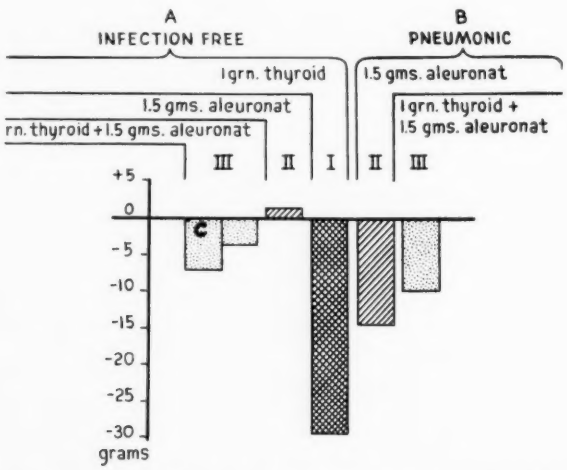

Fig. 3.—A comparative chart showing the average daily rate of weight change in animals of experimental groups I, II, and III. *A*, second series of experiments which were not complicated by intercurrent infection; *B*, preliminary experiments in which pneumonia intervened; *C*, animal 3, the only group III animal to die of this particular treatment.

of accuracy, coincide phase by phase with the cyclic weight changes mentioned above, and that there is an even closer correlation between cyclic lymphocytoses and the weight changes (Fig. 6).

Group III: All but one of the group III animals receiving lethal doses of thyroid substance in conjunction with aleuronat lived for an indefinitely long time. Only animal 3 died during the course of experimentation, but it lived thirteen days. The exact length of time that the other animals could have survived this treatment is not known. Animals 1 and 6 received these drugs for thirty-one days, and animals 2, 4, and 5, for thirty-nine days, without showing evidence of impending death, and in immediately subsequent experiments performed on them, lasting from twenty to sixty days longer, death did not result (Fig. 1).

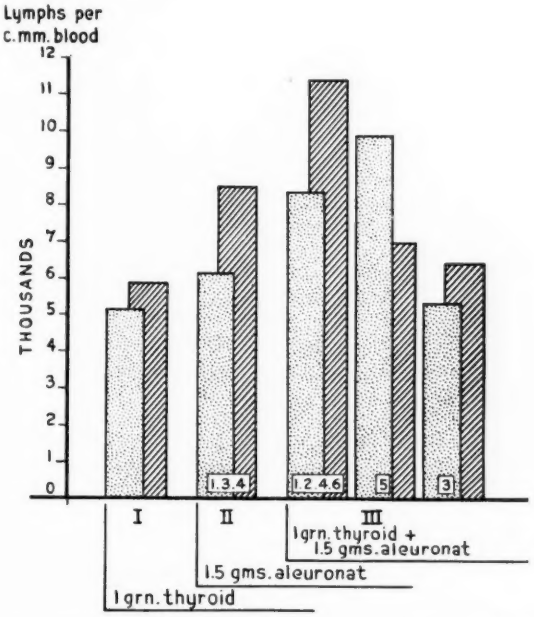

Fig. 4.—A comparative chart showing the average number of lymphocytes per cubic millimeter of blood per experimental day (diagonally lined columns) and the average normal number of lymphocytes per cubic millimeter of blood (stippled columns) of the animals of groups I, II, and III.

The animals in our preliminary group III experiments who contracted and actually died of pneumonia lived 16.5 days (Fig. 1*B*).

In this group the weight changes of every animal were basically alike, being characterized by three common alterations. (1) For a varying number of days each animal progressively lost weight, until about one-third of its original weight was lost. (2) This was followed by an indefinite number of days during which the body weight was stabilized and characterized by minor weight gains and subsequent losses to the basic low-weight level. (3) Weight loss occurred rhythmically in diphasic cycles occupying a varying number of days, usually three to seven. The first phase is characterized by either a slight loss or gain, or no change in weight, and the second by a marked weight loss exceeding the weight changes of the first phase whatever they may be (Figs. 7, 8). The col-

lective daily weight loss of animals 1, 2, 4, 5, and 6 was 3.5 Gm. Animal 3, which died in thirteen days, lost 6.9 Gm daily, which is double the weight lost by the others (Fig. 3*A*).

The average daily weight loss of our preliminary group III animals was 10 Gm. (Fig. 3*B*).

The number of red blood cells with daily variations increased above normal (Figs. 7, 8). There was much daily variation in the total number of white blood cells. The average white blood cell count of animals 1, 2, 4, and 6 during the course of experimentation was only 6 per cent lower than normal. Alterations in the leucocytic formulas of these animals showed the closest agreement possible. For the first few days (one to five) the number of lymphocytes remained near the normal level or slightly below it, but this period was followed by a marked rise in the lymphocytes so that, inclusive of both periods, the average number of lymphocytes per experimental day was 35.7 per cent above normal (Figs. 4, 7, 8). The neutrophiles increased slightly during the first two days of experimentation, but they later decreased considerably below normal to a stabilized level. The average number of neutrophiles per experimental day was 36.8 per cent below normal (Figs. 7, 8).

Animal 5, although unknown to us in the beginning, had a preexperimental existing pathology. Its total white blood cell count before experimentation was 12,960, of which 76.5 per cent were lymphocytes and 19.5 per cent were neutrophiles. During the course of experimentation, the total white blood cell count was on the average 35.7 per cent below this animal's normal, but this was due mostly to a 29.3 per cent (in absolute terms 2,914 cells per cubic millimeter) decrease in the number of lymphocytes, and to a lesser extent by a 47.7 per cent (1,320 cells per cubic millimeter) decrease in the neutrophiles. After suspension of the experimental feeding, the blood picture returned to the normal for this animal.

The total white cell count of animal 3 was reduced 12.2 per cent during the course of study. Alterations in the leucocytic formula were like those of animals 1, 2, 4, and 6 for the first six days, but on the seventh day the neutrophiles increased to near the normal number, and for the remainder of the time that this animal lived, the leucocytic formula was practically normal.

In all animals of this group lymphocytoses occurred rhythmically in diphasic cycles which coincided phase by phase with concurrent cyclic weight changes (Fig. 8).

Our experimental method was built upon the proposition that if lymphocytes were functionally related to a hyperthyroid condition, an added concurrent stimulation of lymphopoieses and lymphocytosis in an experimental hyperthyroid state should mitigate the effects of this hyperthyroid state. Experimental bases of comparison were established by the experimental series of groups I and II.

Our group II series of experiments showed that aleuronat when taken by mouth induces a generalized lymphoid reaction in which a persistent and high lymphocytosis is characteristic of the peripheral blood. The exact modus operandi of aleuronat in effecting lymphocytogenesis is unknown, but it seems

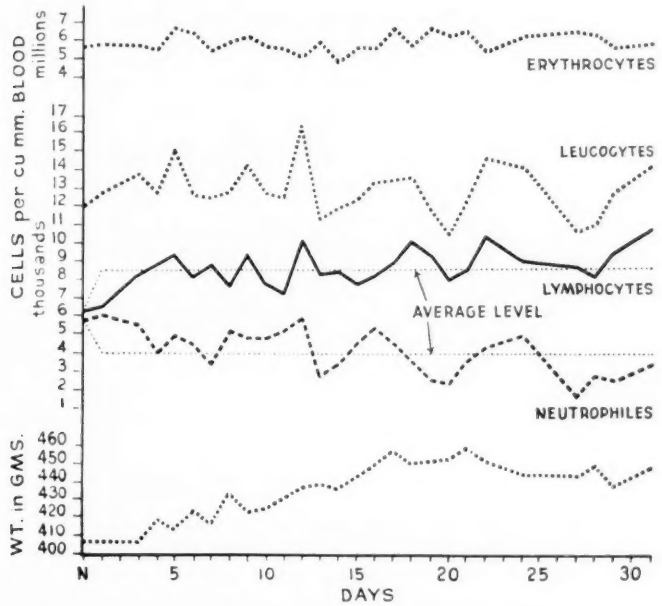

Fig. 5.—A composite graph of the daily alterations in weight and blood picture of animals 1, 3, and 4 of group II which were fed aleuronat.

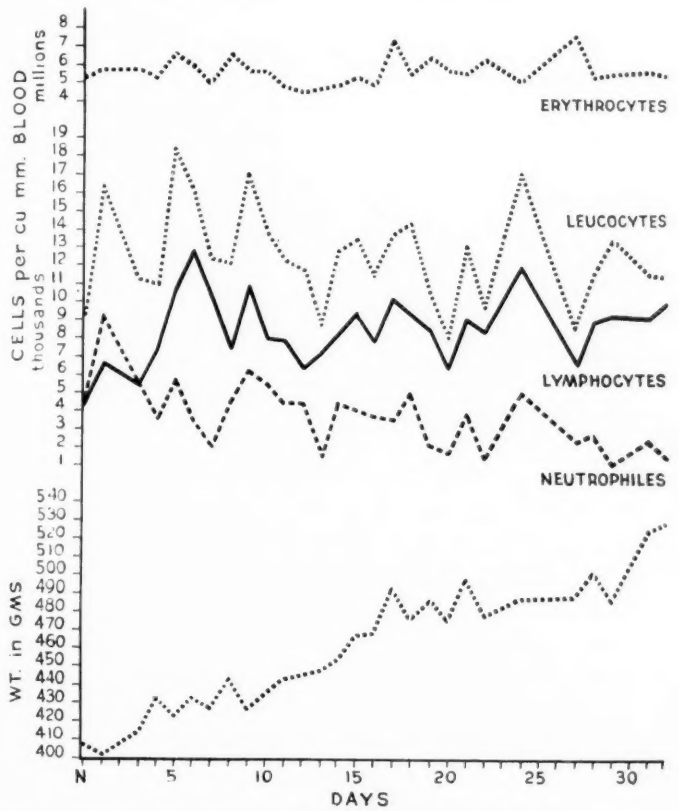

Fig. 6.—A graph showing the daily alterations in weight and blood picture of animal 3 of group II.

to be something of a toxic reaction. If weight loss may be used in this instance to indicate toxicity, it is evident that the aleuronat in the amounts given was only slightly toxic because the animals in this series actually gained weight during the greater part of these experiments which lasted thirty-two days, and when weight loss did occur, it was so slight and of such short duration that it seems negligible and to have little bearing on the final interpretation of our results.

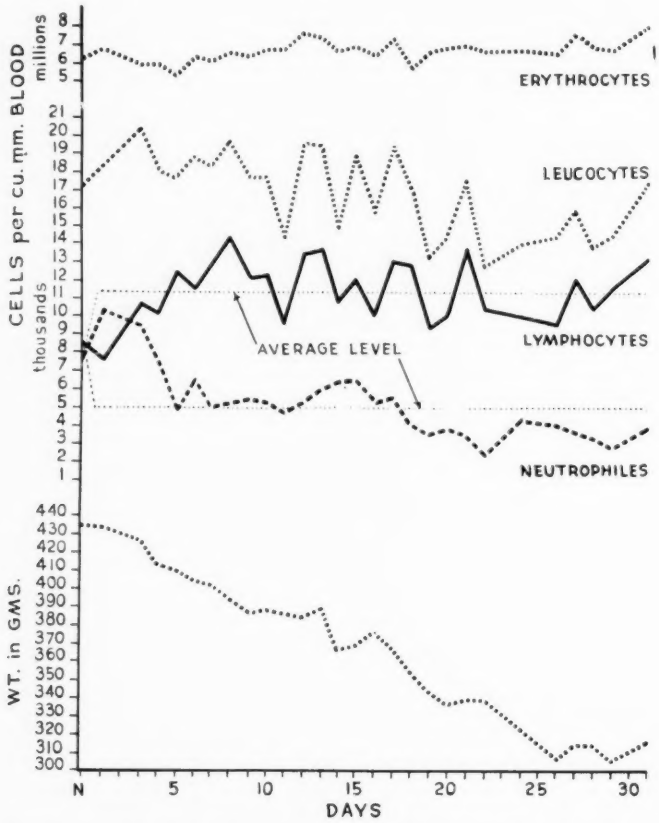

Fig. 7.—A composite graph showing the daily alterations in weight and blood picture of animals 1, 2, 4, and 6 of group III which were fed thyroid and aleuronat.

Our group I series of experiments showed that the hyperthyroid state induced by the daily administration of 1 grain of thyroid substance causes death after three days of treatment; that death is preceded by marked daily weight losses, and by a progressive decrease in the degree of lymphocytosis on days subsequent to the first experimental day which varies directly with the amount of thyroid given. The latter phenomenon is subject to many interpretations, but we believe that the lymphocytes are "used-up" in some manner faster than they can be produced. We rather doubt that the reduction in the number of lymphocytes in peripheral blood is due to a premature death of the lymphocytes because of an increased metabolic effect of thyroxin, because thyroxin seems unable to increase metabolic activity of lymphoid tissue (Anselmino, Eichler, and Schlossmann[9]).

With this knowledge of the individual effects produced by the daily administration of 1 grain of thyroid and 1.5 Gm. of aleuronat, an interpretation becomes possible of the results obtained in our group III series of experiments, in which both of these drugs in the amounts just indicated were administered jointly. The results of these group III experiments showed that, although 1 grain of thyroid substance daily did induce a marked hyperthyroid state in these

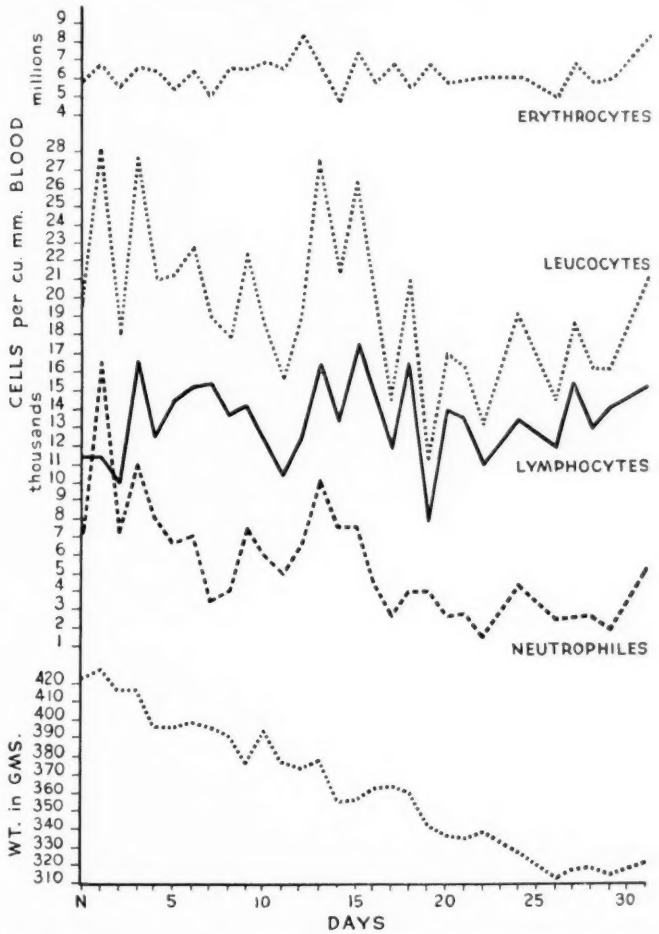

Fig. 8.—A graph showing the daily alterations in weight and blood picture of animal 1, group III.

animals when administered in conjunction with aleuronat, the severity of the hyperthyrosis was considerably less than that produced when this amount of thyroid substance was given alone, as in group I. In view of the facts that all the animals in group III lived longer, and, with the exception of one animal, lived indefinitely longer than did those of group I; and that the rate of weight loss experienced by these animals was but a fraction of that of the animals of group I; and that even the animals of our preliminary group III experiments who contracted and actually died of pneumonia lived five times longer and lost weight more slowly than the animals of Group I; the inevitable conclusion is,

that the mitigation of the known potential fatal effects of 1 grain of thyroid substance when taken daily must have been due to the lymphocytogenic effect of the aleuronat, and that the lymphocytes constituted the means by which this mitigation was accomplished. It would seem that this inference is correct for several additional reasons. (1) In all group III animals there was an almost direct correlation phase by phase of cyclic lymphocytoses with cyclic weight losses which showed that when the number of lymphocytes increased, the rate of weight loss decreased; and when the number of lymphocytes decreased, the rate of weight loss increased. In group II such a correlation is also evident, for the rate of weight gain is greater when the number of lymphocytes increases; and conversely, when the number of lymphocytes decreases, the rate of weight gain decreases. (2) The only death occurring among the group III animals involved an animal whose hemopoietic system failed to respond as expected on the basis of the group II experiments to aleuronat stimulation. (3) Animal 5, group III, which before experimentation had an existent pathology requiring a high lymphocytosis was able to mitigate completely the fatal effects of lethal doses of thyroid substance even in the face of a persistent and lower degree of lymphocytosis which occurred during the course of experimentation. However, the total lymphocyte count of this animal, in spite of this reduction, was above that of the animals of group I and above that of animal 3 of group III which died because of a poor lymphocytic response. (4) In supplementary experiments on two animals in group III to test the effects of continued thyroid feeding alone, a lymphocytosis was maintained at a high level. (5) Death occurred in every instance when the time rate and degree of lymphopoietic response was less than the time rate and degree of development of the thyrotoxicosis. (6) It would seem further that the mitigation was due directly to the lymphocytes rather than to any reaction between aleuronat and thyroxine, or to the action of antihormonal substances, because two animals of group III which were fed thyroid in the absence of aleuronat for thirty days or longer in supplementary experiments showed no augmented thyrotoxic symptoms; and when the dose of thyroid was suddenly increased, death occurred as promptly as it did in those animals of group I.

CONCLUSIONS

(1) Aleuronat when taken by mouth, and in doses of 1.5 Gm. daily, induced a characteristic lymphocytosis in the blood of guinea pigs weighing between 300 and 500 grams each. This lymphocytogenic effect of aleuronat may have been due to the fact that the drug was slightly toxic.

(2) The daily feeding of 1 grain of desiccated thyroid substance to half-grown guinea pigs produced a thyrotoxicosis which was fatal in from two to four days.

(3) The daily feeding of 1.5 Gm. of the lymphocytogenic drug, aleuronat, in addition to a daily lethal dose (1 grain) of thyroid substance to half-grown guinea pigs either prevented completely or delayed the development of a fatal thyrotoxicosis. The evidence indicated that the living lymphocytes constituted the means by which this mitigation of the known potential fatal effect of daily 1 grain doses of thyroid was accomplished.

(4) The lymphocytes in thyrotoxic lymphocytosis are performing a function directly related to the alleviation of the thyrotoxicosis.

(5) It would further seem that the lymphocytic infiltration of toxic thyroid glands is an expression of this lymphocytic function.

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A STATISTICAL STUDY OF TEMPERATURES IN HYPOGLYCEMIC COMA*

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INTRODUCTION

THE use of hypoglycemic comas in the treatment of schizophrenic patients presents such striking clinical features that there is a temptation to generalize from observations during the treatments. This is particularly so if the observation is repeated in a number of different cases. One of the most significant physical changes induced by insulin hypoglycemia is low body temperature, the reading sometimes being below 90 degrees. The depth to which these temperatures will drop seems to vary greatly, but observations suggest that they bear some interesting constant features. It was noticed for instance that in some cases patients whose temperatures dropped very low during the early treatment periods did not reach such a low point during later treatments. It seemed, therefore, to be of some interest to determine whether a statistical analysis of a sufficient number of cases would yield any reliable date concerning this rise. We wished to determine whether it bore some definite relationship to the outcome of the treatment; whether it was related to the dosage of insulin used, or whether it was an individual expression of the patient treated.

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Patients were observed carefully during each shock period and individual manifestations of the shock were recorded on a chart previously described.[7] Dosage was regulated so that the patient was in coma at the end of three hours and had one hour of this for each treatment after the first ten treatments, which were usually less prolonged.

If the temperature drop during hypoglycemia is due to the size of the insulin dose, it may be that the compensatory rise depends on the adequacy of the autonomic centers to meet this strain. There is much to indicate that schizophrenic patients lack the ability to respond with such homeostatic mechanisms.[3-5, 8, 9] That hypoglycemia induces a compensatory sympathetic activity has been indicated by many authors.[1, 6, 10] We might expect, therefore, that the better patients would tend to be better able to combat the temperature drop; that as recovery took place the coma temperature would be closer to the normal temperature and the temperature would tend to keep constant regardless of the size of insulin dosage.

METHODS

In these studies the insulin injections were given at 6 A.M. and the patients were given glucose at about 10 o'clock or later. The temperature was taken at 10 o'clock before termination by means of glucose gavage. At this time most of the patients were in coma. Rectal temperatures were used throughout this study.

The daily 10 A.M. shock temperature records of 36 patients who recovered after insulin shock were studied. This group was considered "A" recoveries and will be referred to as group A throughout this study. Twenty patients who failed to show any improvement and are, therefore, designated as group D were likewise studied.

The first forty-five days were studied in each case since many patients were not treated longer than this. The first statistical study consisted of obtaining a mean of the 10 o'clock temperature, for all patients of each group, and for each day of the forty-five days.

The range of daily means for the coma temperatures of group A were from 96.79 for the first day of treatment to 97.02 for the forty-fifth day of treatment. Although the means did not show a steady course, there was a definite trend upward. In the D group, although the mean for the first day was 97.04 and for the last day 96.7, the daily means fluctuated so unsteadily that there was no constant trend.

When the temperatures were grouped for periods of one week or five days, the same trend was noticed. The averages for group A for the first and ninth weeks were 96.67 and 96.78, respectively. For the D group these means were 96.53 and 96.54. It must be remembered that each of these means represents five days' average for a large number of different patients. Although the trend upward in the A group may certainly be considered greater than that of the D group, there is statistically no reliable difference between the two.

A study of the units of insulin used for each treatment was then considered. Examining the amount of insulin used in the first dose (the shock dose), we find a mean of 104.03 for the A group, and a mean of 112.22 for the D group.

These differences are not reliable statistically. The shock doses of the two groups are approximately the same. Obtaining the means for the insulin given to the A group for each of forty-five treatments, we found the mean to be 104.03 on the first day of treatment and 76.1 on the forty-fifth day. In the D group these amounts were 112.22 to 83.33, respectively. There is a reliable difference between the dosage used at the beginning and end of treatment in group A, the insulin used being greatly diminished. In the D group the dosages were also diminished, although the difference is not as constant for all members of the group. On the whole, moreover, we note that the total insulin used on the A group for each of the forty-five days of treatment is less than that used on the D group.

TABLE I

DAILY AVERAGES OF INSULIN DOSE AND TEMPERATURES IN HYPOGLYCEMIC SHOCK

TIME OF TREATMENT	TEMPERATURE		INSULIN	
	MEAN	SIGMA	MEAN	SIGMA
Group A, 36 Patients				
1st day	96.79	0.98	104.03	44.5
45th day	97.02	1.17	76.11	42.9
1st week	96.67	1.22	110.7	46.2
9th week	96.78	1.33	75.2	41.7
Group D, 20 Patients				
1st day	97.04	1.10	112.22	61.4
45th day	96.7	1.61	83.33	54.8
1st week	96.53	1.38	118.7	68.7
9th week	96.54	1.71	83.1	52.7

It might appear that the rise in temperature observed in improved patients in the course of the treatment may be due to the greater decrease in the amounts of insulin used in this group during the treatments as compared to that of group D. In order to see whether this is true, partial correlations between day of treatment, insulin dosage, and coma temperature, were made. These correlations were found to be so low that it can be said that no correlation exists between insulin dose and temperature.

TABLE II

PARTIAL CORRELATIONS BETWEEN DAY, AMOUNT OF INSULIN, AND 10 O'CLOCK TEMPERATURE

	Group A, 1,364 Treatments		
	(1) *Day*	(2) *10 o'clock Temperature*	(3) *Insulin*
Mean	23.15	96.6	77.3
Sigma	12.48	1.27	38.6
Correlation between	(1) and (2) = 0.13 ± 0.02	(2) and (3) = 0.02 ± 0.02	(1) and (3) = −0.27 ± 0.02
	Group D, 796 Treatments		
	(4) *Day*	(5) *10 o'clock Temperature*	(6) *Insulin*
Mean	22.67	96.46	94.7
Sigma	12.75	1.61	60.8
Correlation between	(4) and (5) = 0.02 ± 0.02	(5) and (6) = 0.06 ± 0.02	(4) and (6) = 0.20 ± 0.02

A study of the temperature and insulin dose, considering all days for all patients of the A group compared to those of the D group, reveals some striking findings. The temperatures of those in group A were higher than those in group D. The reliability of this difference is 2.09, indicating a significant difference, which probably would be proved to be statistically reliable if more cases were considered. The amount of insulin used all forty-five days for patients of group A was less than that used in patients of group D, and this difference is statistically reliable.

DISCUSSION

We noticed that patients who recovered from schizophrenia after undergoing insulin shock therapy (group A patients) required less insulin during the course of treatment. This may suggest that patients requiring lower doses to induce shock may have a better chance of recovering than those requiring larger doses. On the whole, moreover, the group A patients do not reach as low temperature levels as do the patients who do not improve. Of interest is the demonstration in these studies that the group A patients show an increasing temperature during the course of treatment, with a tendency to return during insulin coma to normal temperature levels. The possibility suggests itself that this indicates an improvement in the homeostatic functions of these patients. That such improvement occurs has been suggested by other authors as demonstrated by improved blood pressure and other signs of cardiac functions.[2] It would be interesting if the probability of a good remission could be predicted during treatment by upward rise in the coma temperature curve. At any rate, the findings presented here indicate that the determination of temperature during insulin shock may be of definite importance beyond mere interest, and that the supposition that the temperature level observed during hypoglycemia is merely dependent on dosage of insulin is unfounded.

SUMMARY

1. Statistical studies of rectal temperatures taken during insulin coma are reported.

2. The range of daily means for 36 recovered patients showed a trend upward. Twenty unimproved patients showed no such rise.

3. Initial insulin shock doses were the same in both groups. There was a definite decrease in the daily insulin dosage used in the course of treatment, this decrease, however, being more constant in the A group.

4. We note, moreover, that the total insulin used on the A group is less than that used on the D group.

5. The rise of temperature in the course of treatment in the A group is not due to the decrease in the insulin dosage.

6. The temperature of the group A on the whole was greater than that of the group D.

7. The amount of insulin used for the group A patients for all the treatments was less than that used on the group D patients.

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SEROLOGIC DIAGNOSIS IN HISTOLOGICALLY PROVED CHRONIC SYPHILIS*

An Analysis of 424 Necropsies

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ONE frequently finds contradictory figures given as to the percentage of positive serologic tests in cases of various types of chronic syphilis. It was for this reason that it was decided to analyze the autopsy protocols of the Detroit Receiving Hospital for the period from January, 1928, to July, 1940, in order to determine the incidence of positive serologic reactions in the various forms of proved autopsy syphilis.

The serologic tests performed were as follows: previous to November, 1933, Kolmer and Kahn tests; from November 1, 1933, to January 1, 1934, Kolmer, Kahn, and Kline tests; and from January 1, 1934, to July 1, 1940, the Kline test was used as an exclusion test, and Kahn tests were performed on those sera which gave positive Kline reactions.

No attempt was made to determine the relative merit of the serologic tests employed. If any one test was positive, it was considered sufficient reason for recording the serology as positive for syphilis. Results were reported as positive, negative, or doubtful.

*From the Pathological Laboratories of Receiving Hospital, Detroit.
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In this study of autopsy protocols for the period mentioned, 555 persons with microscopically proved syphilis were encountered. However, of this group, only 424 patients had an accompanying serologic report. The remainder died in the admitting room or on the wards shortly after admission, and no opportunity was afforded of obtaining a specimen of blood.

TABLE I

SEROLOGIC REPORTS

	CASES	PER CENT
Positive	321	75.7
Negative	82	19.3
Doubtful	21	5.0
Total	424	100.0

Table I shows the serologic findings in the 424 patients included in the study. It is noted that 75.7 per cent of these gave positive serologic reports.

In attempting to compare the figures obtained at the Receiving Hospital with those frequently quoted, one finds that very often there is no common ground upon which this comparison can be made. Most of the figures cited in the literature are based upon studies of groups of living patients, whereas, the cases covered in this report were all proved by microscopic examination of autopsy material. Kolmer[1] contends that in 80 to 100 per cent of chronic syphilis the serology is positive, and Stokes[2] states that he found 67 per cent of 252 persons to have positive serology. However, these figures were not compiled from autopsied material.

In this series it is seen that in 82 of 424 persons the serology was negative; a further analysis of this group brought out the interesting fact that in 60 per cent of the 82 cases, only syphilitic aortitis was found at autopsy. This leads to the question: Does the presence of a positive serologic reaction depend upon at least a certain minimal involvement of the organs and structures, or do the accepted pathologic criteria of what constitutes syphilitic aortitis need revision? If the answer to the first part of the question is in the affirmative, it would seem when there is syphilitic involvement of several organs the percentage of positive laboratory tests for syphilis should be higher. Table II shows the results of such an analysis.

TABLE II

RELATION BETWEEN EXTENT OF INVOLVEMENT AND PERCENTAGE OF POSITIVE TESTS

	CASES	PER CENT
Only one organ involved (usually syphilitic aortitis):		
Positive	206	71.0
Negative	66	23.0
Doubtful	19	6.0
More than one organ involved:		
Positive	115	86.0
Negative	16	12.4
Doubtful	2	1.6

It can be seen that the incidence of positive tests in the group of patients with multiple involvement was 21 per cent higher than in those with a single organ involved, and that there was 14 per cent more in the former group

who gave a positive reaction than in the total group of 424 persons. This finding is far from conclusive, but, nevertheless, gives some basis for the contention that positive serologic evidence of syphilis is somewhat dependent upon the extent of demonstrable pathologic involvement.

CARDIOVASCULAR SYPHILIS

Cardiovascular syphilis formed a major portion of the material studied. Some evidence of cardiovascular involvement was found in a total of 335 patients, or 79.0 per cent. This figure is about 33 per cent higher than that given by Symmers,[3] who found evidence of vascular system syphilis in only 56 per cent of the 314 cases he studied. In this series syphilitic aortitis was the most common lesion encountered, occurring in 303 patients, or 71.4 per cent of the total (424 patients), a figure which is closely comparable to the one given by Cecil,[4] who maintains that syphilitic aortitis is present in 70 per cent of persons with chronic syphilis.

In analyzing the serologic reports of the cardiovascular syphilis group, it was found that 71.6 per cent gave positive serology. This figure is considerably below the one given by White,[5] who states, that "the serum reaction for syphilis is generally positive, and strongly so in cardiovascular syphilis; sometimes in approximately 15 per cent of the cases it is negative." Kolmer[1] claims that in chronic cardiovascular syphilis 80 to 96 per cent of patients will give positive serology by his method. Moore[6] found 85 to 95 per cent of his clinical cases to be positive. Christian[7] found that about 80 per cent of patients with syphilitic disease of the aorta gave a positive Wassermann reaction.

SYPHILIS OF ALIMENTARY TRACT

Thirty-five patients (8.2 per cent) had a diagnosis of some form of syphilitic involvement of the digestive tract. Table III shows the distribution of such lesions.

TABLE III

SYPHILIS OF ALIMENTARY TRACT (35 CASES)

	POSITIVE	NEGATIVE	DOUBTFUL
Liver	21 (80.7%)	4 (15.3%)	1 (4.0%)
Pancreas	7 (77.7%)	2 (22.3%)	0
Esophagus	1	0	0
Midgut	1	0	0
Cecum	1	0	0

Syphilis of the liver constituted only 6.1 per cent of the 424 patients in this series. Symmers[3] found the liver to be involved in 33 per cent of cases showing post-mortem evidence of syphilis. Brockbank[8] found the above to be true in 58 per cent of his cases. Flexner[9] found 88 cases of hepatic syphilis in 5,089 autopsies (1 per cent). McCrae[10] demonstrated 46 cases in 3,300 autopsies (1.3 per cent). The reason for the marked disparity between the figures quoted and those obtained in this series is not apparent.

Since the number of cases of syphilitic involvement of the liver[26] is comparatively small, the percentage figures obtained are indicative rather than conclusive. A total of 80.7 per cent had positive serology. McCrae and Caven[11] found

that 80.4 per cent of 41 persons with syphilis of the liver, which had been successfully diagnosed and treated, gave positive Wassermann reactions originally. O'Leary, Greene, and Rowntree[12] found that 90 per cent of their patients with hepatic syphilis had positive Wassermann reactions. Their diagnoses were made for the most part on the basis of clinical impressions and the effect of therapeutic tests.

CENTRAL NERVOUS SYSTEM SYPHILIS

Numerically, the group with syphilis of the central nervous system was second only to that showing cardiovascular syphilis. One hundred and thirty patients, or 30.6 per cent of the total, were found to have some form of syphilitic involvement of the central nervous system. No doubt this figure is much lower than the actual incidence of involvement of the central nervous system, since permission for examination of the cranial cavity of the patient was frequently not obtained. Of this number, 85.4 per cent had positive serology. Included in this group were 57 patients with general paresis, 87.7 per cent of whom had positive serology.

The figures given in the preceding paragraph differ from those given in reports by some well-known authors. Cecil[4] states that it is unusual and rare for the serology to be negative in syphilis of the central nervous system. Grinker[13] found the blood Wassermann positive in 65 per cent of all patients with neurosyphilis and that it may fall as low as 50 per cent in the latent phases of the disease. Kolmer's[1] figures ranged between 70 per cent and 100 per cent in his groups of patients with syphilis of the central nervous system. In general paresis, Grinker states, the blood serology is 100 per cent positive. Moore[6] found it 90 to 95 per cent positive. These figures are definitely higher than those found in the group of cases covered in this analysis.

SUMMARY

1. A total of 424 patients with microscopically proved syphilis encountered at autopsy were analyzed statistically. All of these had accompanying serologic examinations.

2. Of the entire group 75.7 per cent had positive serology.

3. The patients with multiple organs involved gave 21 per cent more positive reactions than those with only one organ involved.

4. A total of 335 patients (79.0 per cent) showed some evidence of cardiovascular involvement. Of these 71.6 per cent had positive serology.

5. There were 26 cases of hepatic syphilis. This amounted to only 6.1 per cent of all the cases. In 80.7 per cent of these the serology was positive.

6. One hundred thirty persons exhibited central nervous system involvement, and 85.4 per cent had positive serology. In 87.7 per cent of 57 persons with paresis the serology was positive.

CONCLUSIONS

1. The percentage of positive blood tests for syphilis for the entire group studied did not differ appreciably from that usually reported.

2. The percentage of positive reactions in cardiovascular syphilis was about 10 per cent lower than the figures that are usually given.

3. Demonstrable hepatic syphilis occurred 25 to 50 per cent less frequently than in many other series.

4. The percentage of positive tests in hepatic syphilis coincided closely with the experience of others.

5. The percentage of positive tests in general paresis was definitely below that found in other series.

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THE DIAGNOSIS OF PERIPHERAL ARTERIAL OBSTRUCTION*

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TRADITIONAL methods of examination for the diagnosis of peripheral arterial obstruction are becoming inadequate and uncertain because of the demand for more reliable and accurate information in this field of medicine. Sole reliance upon palpation of the dorsalis pedis pulse as the standard procedure in the diagnosis of circulatory disturbances in the lower extremities is rapidly losing its former worth.

Manual palpation of peripheral pulses may be misleading because of the following variable factors involved: (1) The skill and experience of the examiner. (2) Anatomical deviation in position of the peripheral arteries. (3) Edema or excessive adiposity of the extremities.

These objections apply not only to the dorsalis pedis pulse but also to the posterior tibial and popliteal as well. The familiar sight of a group of physicians in a vascular clinic disputing the presence or absence of a dorsalis pedis pulse is sufficient argument for the claim that individual skill and experience play a considerable part in this diagnostic procedure.

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Occasional instances of congenital absence of the dorsalis pedis artery have been observed. In these cases there is usually compensatory enlargement of a branch of the peroneal artery which produces pulsation in the lateral portion of the foot.

In the presence of edema of the feet either from cardiorenal or from infectious cause, the dorsalis pedis and posterior tibial pulses may be completely impalpable, although the arteries are patent. In obese patients similar difficulties may be experienced, not only with the arteries of the feet but with the popliteal as well.

Palpation of the popliteal artery is attended with considerable difficulty, and often requires various acrobatic manipulations in order to feel pulsation of the vessel adequately. The status of the digital and plantar circulation of the lower extremities is not accurately revealed by palpation of the dorsalis pedis or posterior tibial arteries alone. If both of these vessels are felt in an extremity there is no assurance that the arterial tree distally is also patent. To ascertain this fact other tests must be utilized.

The importance of the detection of peripheral arterial disease in its early stages is obvious. Any method of examination which will reveal the presence of organic changes in the most distal parts of the extremity should be utilized more frequently than has heretofore been the rule. At the same time the physician who has relied traditionally solely upon palpation of the dorsalis pedis artery should realize the limitations of this method.

It is well to re-emphasize the fact that an important diagnostic point in the organic peripheral arterial diseases is the ability to detect the organic changes. The presence or absence of superimposed vasomotor disturbances, particularly vasospasm, is of secondary importance in diagnosis, the primary consideration being the detection of structural arterial disease. The tragedy of delayed diagnosis was recently illustrated in a case of thromboangiitis obliterans in a physician. About eight years ago he experienced symptoms in his legs which suggested to him the possibility of thromboangiitis obliterans. A physician was consulted who, after feeling both dorsalis pedis pulses and using no other diagnostic procedure, concluded that there was no evidence of arterial disease and that smoking might be resumed. A few years later the physician submitted himself for a blood count, for the determination of which it was necessary to puncture the tip of the right index finger. The trauma thus induced resulted in a gangrenous ulcer which did not heal until a year and a half later. At the first sign of gangrene the patient presented himself for an opinion. The lower extremities were examined and revealed definite right plantar ischemia in addition to poor dorsalis pedis pulses and excellent posterior tibial pulses. Oscillometric readings were 3.0 at the left ankle and 2.0 at the right ankle. Although both radial and ulnar pulsations could be felt, there was moderate ischemia of the fingers of both hands on elevation and exercise. There was a gangrenous ulcer at the tip of the right middle finger about 1 cm. in diameter. The patient refused to stop smoking, and about a year later no pulses could be felt in either foot and the oscillometric reading at the left ankle had decreased to zero. Extensive gangrene of the right index finger soon developed as well as gangrenous lesions of the left big toe and heel.

It is clear that had the diagnosis been made at the first examination, and had the patient been sufficiently impressed with the importance of complete cessation of smoking, the present situation could have been avoided. The assurance of the original examiner was so great that there was no arterial disease because of the bounding dorsalis pedis pulses that the patient was offered a package of cigarettes and told to smoke as much as he pleased.

In 1929 I[1] proposed a new test for the determination of arterial occlusion in the distal vessels of the extremities. This procedure, based on earlier observations of Oehler and Parkes-Weber, has proved so reliable over a period of fifteen years that it must be considered of diagnostic importance and as an obligatory part of the examination before the diagnosis of arterial disease is ruled out. Kramer,[2] from his large experience at the Philadelphia General Hospital, says "a positive plantar ischemia is significant. Our experience with this simple procedure has been that when positive findings are obtained, the other function tests will usually indicate evidence of an impaired peripheral circulation." Again, "we have found that a positive plantar ischemia has nearly always been associated with occlusive vascular disease." Lewin[3] also considers the test an important part of the routine examination for determining the circulatory efficiency of the lower extremities.

In carrying out the test the patient lies recumbent on the examining table, and the legs are elevated to an angle of about 60 degrees, supported on a stool or some other arrangement. While in this position the ankles are flexed and extended repeatedly for about a minute. During and immediately after the maneuver the soles of the feet are carefully examined in a good even light. In every instance of organic arterial disease, whether due to thromboangiitis obliterans, or arteriosclerosis obliterans, a characteristic pallor of the sole of the affected extremity will become apparent either during the motion of the feet or immediately afterwards. The pallor, or plantar ischemia, is usually in direct proportion to the degree of obstruction present. In incipient cases, particularly those with patent dorsalis pedis and posterior tibial arteries, the ischemia may be difficult to detect by the novice, but experience will soon help in judging relative differences in color.

In the more advanced cases no difficulty should be experienced in detecting plantar ischemia. A cadaverous pallor, which may involve the entire foot as high as the ankle or farther, becomes apparent almost immediately.

The same principle of inducing ischemia as a diagnostic method may be applied to the upper extremities. Elevation of the hand above the head with alternate opening and closing of the fingers will produce, after a few moments of this motion, a transient ischemia of the digits or hand deficient in blood supply. This test may be positive in incipient cases of organic arterial disease in which the radial and ulnar pulses can be felt. It is of more practical value than the modification proposed by Allen[4] which merely serves to distinguish between closure of the radial or ulnar artery. The test here described is more suitable for detecting early involvement of the digital arteries of the upper extremities.

Temperature changes are usually very pronounced in organic arterial disease even in the earliest stages. As a rule, the ischemic extremity is cor-

respondingly colder than the uninvolved limb. Where the condition is bilateral both will be colder than usual, with proportionate differences in coldness dependent upon the degree of occlusion present. For ordinary clinical diagnosis instrumental measurements are not necessary for temperature determination, the palm or back of the hand being sufficiently sensitive. For precise measurements of the skin temperature, the Dermalor[5] is preferred.

Fig. 1.—Hellige-Samuels pulsimeter.

OSCILLOMETRY

The observation of Cawadias in 1912 that the Pachon oscillometer was admirably suited to the study of arterial patency in the extremities opened up a new and interesting field. Visual observation of arterial pulsation was made available so that quantitative measurement of the pulse amplitude in the extremity could be easily determined. Furthermore, pulsation could be detected at points where by manual palpation the arteries were not accessible. The examining cuff could be placed at any point on the limb, and oscillations were recorded at that particular level, whereas by the manual method one is limited to only a few areas.

While the clinical diagnosis of arterial obstruction can be made without instruments, it is also true that a more accurate picture is conveyed by oscillometric determinations than by purely clinical methods alone. Other valuable information can also be obtained from the oscillometer. If one remembers that the pulsation obtained with the instrument represents the expansion of the main vessels only, deductions can be made therefrom concerning the state of the collateral circulation in the extremities in an indirect way. For instance, a negative reading at the ankle level indicates complete obstruction of the main arterial channel. This being the case, it follows, therefore, that the collateral circulation in the extremity must be very small. On the other hand, a positive reading at the same level indicates only partial occlusion of the main vessels and a correspondingly better collateral circulation. For prognostic study such observations are extremely important because a zero reading at the ankle level in a case

of arterial disease would naturally convey the impression that the ultimate recovery of gangrene, for instance, would be doubtful because of the poor collateral circulation. On the other hand, a positive reading gives more encouragement to conservative therapy with better chances for eventual healing.

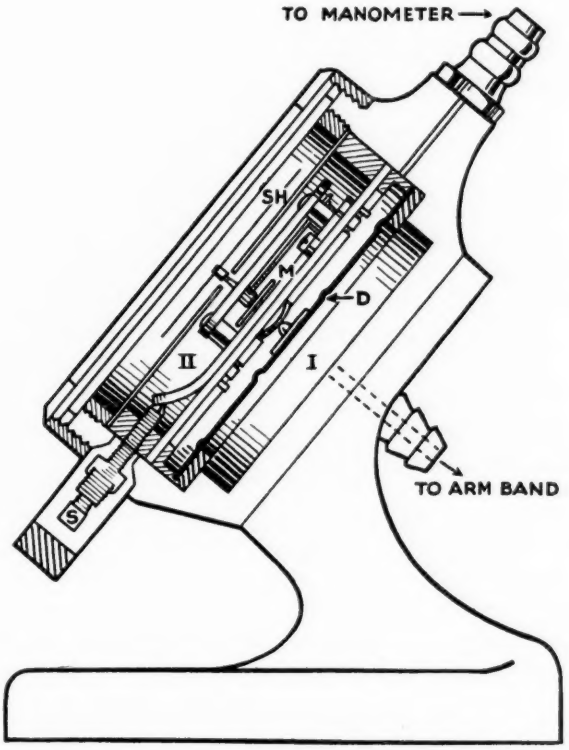

Fig. 2.—Schematic drawing to show construction of pulsimeter. *D*, aneroid diaphragm; *M*, mechanism for transmitting pulsation to indicating hand *SH*; *S*, set screw for adjusting indicating hand to zero; *I*, *II*, air chambers separated by diaphragm *D*.

Likewise, in cases without ulceration and gangrene zero readings mean more difficulty in conservative treatment, a longer period of therapy to attain symptomatic relief, and a doubtful outcome as far as ultimate development of gangrene is concerned. Positive readings in these cases mean better therapeutic results and less chance for the development of gangrene.

The oscillometer has thus extended the possibilities in the field of peripheral vascular diseases and should be made more available so that more accurate studies can be made and more practical information can be obtained. One of the great hindrances to the more universal use of the instrument has been its comparatively high cost. To overcome this objection a simplified apparatus has been devised which does not entail a large financial investment. This apparatus, because of its primary purpose of measuring pulsation, is redesignated "pulsimeter."

By means of a short piece of rubber pressure tubing, either an aneroid or a mercury sphygmomanometer is attached to the upper nipple of the pulsimeter. One outlet of the arm band of the blood pressure apparatus is connected to the

rear nipple of the pulsimeter. It is essential that all connections be absolutely tight, so that there will be no leakage of air. If the inflating bulb of the blood pressure apparatus or the rubber arm band or any parts of the connecting tubes are defective, or allow even the slightest loss of air pressure, the pulsimeter will not operate properly.

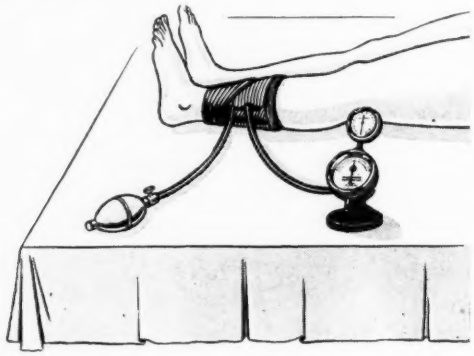

Fig. 3—Method of applying pulsimeter for ankle readings.

In carrying out an examination, it is essential that the patient lie flat, completely relaxed, with the head back and arms at the side. If the patient is not completely relaxed, the slightest muscular movement, such as lifting a hand, will cause deflections of the pulsimeter needle, interfering with the proper operation of the apparatus. The arm band of the sphygmomanometer is carefully applied, as in taking blood pressure, to the part under examination. As a rule, the region just above the ankle gives the most information if the lower extremities are being studied. It is essential that the cuff be applied snugly, not too tightly and not too loosely. Caution must be taken that there are no kinks in the tubing after the cuff is applied.

After all connections are tight and the cuff is properly applied, the escape valve on the inflating bulb of the blood pressure apparatus is closed tightly and the entire system is inflated. By watching the sphygmomanometer, the pressure being applied can be measured. During inflation, the indicating hand of the pulsimeter will rotate a few times. This does not interfere with the operation of the apparatus and should be disregarded until the final inflating point is reached. The pressure in the cuff is increased until it is reasonably sure that the patient's systolic pressure has been exceeded. For routine examinations, it is sufficient to inflate the cuff to about 200 mm. of mercury, as indicated on the sphygmomanometer dial. When this point is reached, the indicating hand of the pulsimeter revolves slowly until the entire system becomes stabilized. It is necessary to wait a few moments for this to happen before readings are taken.

As a rule, a pressure of 200 mm. of mercury will completely obliterate all pulsations; therefore, the indicating hand will not oscillate. In order to estimate the maximum oscillation of the indicating hand, it is necessary to decrease the pressure in the whole system by about 10 mm. at a time. This is done by partially releasing the air escape valve on the inflating bulb. With each deflation, the indicating hand may again revolve once or twice. It is important

to allow the hand to stabilize after each reduction in pressure in order to obtain a reading. As the pressure in the apparatus is decreased in stages of 10 mm. of mercury, the oscillations of the indicating hand increase in amplitude, until a point is reached corresponding to the patient's systolic pressure. When this level is obtained, the maximum oscillation known as the oscillometric index can be read.

It is not necessary that the indicating hand remain at the zero point either before, during, or after an examination. If all oscillations are either to the right or to the left of the zero point, direct readings can be obtained. If the hand swings to both sides of the zero point, it is necessary to add the readings thus obtained to determine the oscillometric index. In other words, if the hand swings from 2 on the left side to 3 on the right, the oscillometric index is 5. Likewise, if the indicating needle swings from 2 on the left side to 7 on the right side, by subtraction an index of 5 is obtained. Similarly, if the needle swings from 2 on the left side to 7 on the left side, the oscillometric index is 5. An irregular pulsation will, of course, give irregular readings. In such a case, the maximum oscillation is recorded.

Interpretation of Oscillometric Readings.—It must be understood that the significance of oscillometric readings for diagnostic purposes lies not so much in the individual values obtained as in the comparison of readings between opposite extremities at the same level. In other words, if the oscillometric index at one ankle is 5 and at the other, at the same level, it is 2, this is evidence of abnormal arterial circulation in the extremity with the lesser reading. On the other hand, readings of 2 in each extremity at the same level may be normal for that particular individual. The normal range of readings in the extremities is as follows: just above the ankle, 1 to 10; calf, 3 to 10; popliteal region, 3 to 10; thigh, 4 to 20. In the upper extremities, at the wrist, 1 to 10; brachial region, 2 to 10; axillary region, 4 to 10. Again, it must be emphasized that this range is very wide and varies considerably with different individuals. It is only by comparing corresponding levels of the extremities that conclusions can be drawn concerning the patency of the arteries.

SUMMARY

Palpation of the dorsalis pedis pulse alone is an inadequate method of diagnosing organic arterial obstruction in the extremities. The test for plantar ischemia should be an essential part of every physical examination. A new simplified inexpensive oscillometer is described.

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SENSITIZATION INDUCED BY TETANUS TOXOID, ALUM PRECIPITATED

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THE recent publication of Cooke and his associates[1] has served to center attention on the problem of sensitization induced by immunization with tetanus toxoid. In their very interesting study, Cooke and co-workers found that allergic reactions occur particularly after the second injection of alum-precipitated toxoid due to sensitization induced mainly by the peptones of the Berna or Witte type present in the toxoid. They also showed that sensitization to the bacterial products of *Cl. tetani* may take place.

Because of the importance of Cooke's observations we deemed it advisable to investigate this problem to determine (1) the frequency of sensitization as proved by skin tests; (2) the incidence of clinical evidence of sensitization; and (3) the possible existence of any correlation between (1) and (2).

Sixty-six patients that had been, or were undergoing, active immunization against tetanus were subjected to skin tests with two lots of materials. Lot A was prepared by a commercial biological house that employs Berna peptone in the manufacture of its tetanus toxoid, and lot B was supplied by a house that uses Difco proteose in its toxoid. All dilutions of lot A materials were made with Glenney's buffered salt solution. Lot B materials were diluted with sterile physiologic saline solution.

The following testing solutions were used: (1) tetanus toxoid A diluted 1:100 and 1:10; (2) Berna peptone A diluted to contain 0.01 and 0.10 mg. of nitrogen per cubic centimeter; (3) veal broth A diluted 1:10; (4) finished tetanus bouillon A diluted 1:100 and 1:10 (this preparation was a veal Berna peptone broth that had been subjected to all the manufacturing steps employed in the production of tetanus toxoid A, except that it had not been inoculated with tetanus bacilli); (5) tetanus toxoid B diluted 1:100 and 1:10; (6) Difco proteose B diluted to contain 0.01 mg. of nitrogen per cubic centimeter; and (7) tetanus media B diluted 1:100 and 1:10.

All persons included in this study had been treated with tetanus toxoid alum-precipitated A. A few also received intranasal instillations of tetanus toxoid topagen A. They may be classified in eight groups according to their immunization history.

Group 1 consisted of 7 patients on whom skin tests were performed two days before and thirty-two days after they had received the first injection of 1.0 c.c. of tetanus toxoid, alum precipitated.

Group 2 consisted of 29 persons on whom skin tests were done seventy-eight days after they had received the first 1.0 c.c. injection of alum toxoid.

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Group 3 consisted of 4 persons on whom skin tests were done 207 days after they had received the first injection of 1.0 c.c. of alum toxoid. Thirty-two days later they were injected with a second dose of alum toxoid. Recheck skin tests were done thirty-two days after the latter was given.

Group 4 consisted of 3 patients (Nos. 19, 20, and 36) on whom skin tests were done 1,045, 1,544, and 1,479 days, respectively (about three to four and one-half years), after they had each received two doses of alum-precipitated toxoid. A third dose of 1.0 c.c. of alum toxoid was administered to two of them (Nos. 20, 36) fourteen days and one day, respectively, after the first skin tests were done. Skin retests were performed thirty-five and twenty-five days, respectively, after the third injection.

Group 5 consisted of 6 persons. Four of them, patients 17, 18, 30, and 35, had received three injections of alum toxoid (0.5 c.c., 0.5 c.c. and 1.0 c.c.) before the first skin tests were performed. These tests were made 766 to 809 days (about twenty-six months) after the third dose of toxoid was given. A fourth injection of 1.0 c.c. of alum toxoid was administered to the 4 patients fourteen, ten, two, and one day, respectively, after the first skin tests were performed. The skin tests were repeated twenty-eight, thirty-two, thirty-two, and twenty-five days, respectively, after the fourth injection of toxoid. The fifth member of group 5, patient 22, was tested 792 days (about two years) after he had received three 1.0 c.c. injections of alum toxoid. Twelve days later he was given a fourth injection of 1.0 c.c. of toxoid. Skin retests were done thirty-five days later. The last member of the group, patient 21, had received four injections of alum toxoid (0.5 c.c. each) prior to skin testing. Thirty-seven months had elapsed between the last dose of toxoid and the first skin tests. Fourteen days after the tests were performed, this person received a fifth injection of 1.0 c.c. of toxoid. The skin tests were repeated thirty-five days later.

Group 6 consisted of 5 patients that had been immunized by the combined subcutaneous and intranasal routes.[2] Prior to the two daily intranasal instillations of tetanus toxoid topagen, patient 31 had received two injections (1.0 c.c. each); patient 34, 3 injections (0.5 c.c. each); patient 37, 5 injections (1.0 c.c., 0.5 c.c., 0.5 c.c., 1.0 c.c., 1.0 c.c.); patient 38, 3 injections (1.0 c.c. each); and patient 39, 3 injections (0.5 c.c., 0.5 c.c., 1.0 c.c.) of alum-precipitated toxoid. Skin tests were performed on the entire group 210 to 263 days after the last toxoid topagen instillation in the nose. One to four days later they all received an additional injection of 1.0 c.c. of alum toxoid. Skin retests were done thirty to forty-two days after this final injection.

Group 7 included 9 patients, 4 of whom had received two courses of three daily intranasal instillations of tetanus toxoid topagen. Four others obtained the same treatment, except that the second course consisted of only two daily instillations. The ninth person received two courses of two daily intranasal topagen instillations. The entire group was skin tested eighty-four days after the last nasal treatment. Thirty-two days later they were all injected subcutaneously with 1.0 c.c. of alum toxoid. Skin retests were done thirty-two days after this injection.

Group 8 consisted of 3 persons, 2 of whom received a single course of three daily topagen instillations in the nose prior to skin testing, while the third

patient got an injection of 1.0 c.c. of alum toxoid previous to the course of nasal treatment. Thirty-two days after the first skin tests were done, all 3 received an injection of 1.0 c.c. of alum toxoid. Actually 240 and 146 days, respectively, had elapsed between the last intranasal instillation and the final injection of toxoid. The skin tests were rechecked thirty-two days after the last injection.

SKIN TESTS

About 0.02 c.c. of each testing solution was injected intracutaneously, and the reactions were read at the end of fifteen minutes and twenty-four hours. The immediate reactions (fifteen minutes) were described as negative, doubtful, slight, moderate, and marked in accordance with the usual allergic practice.[3] In the instances where half-hour readings were made, the reactions did not materially differ from those recorded at the end of fifteen minutes. The delayed reactions consisted of local induration with papule formation, erythema of varying degrees and swelling. In many cases these reactions did not subside until forty-eight to seventy-two hours had elapsed.

The 7 persons tested before immunization showed negative reactions. Of 40 patients tested one to six and one-half months (largest number done at two and one-half months) after the first injection of 1.0 c.c. of tetanus toxoid-alum precipitated (groups 1, 2, and 3), 20 gave positive reactions of the immediate or delayed type to one or more of the testing solutions, while 20 were negative. The reactions varied in intensity from slight (+) to marked (++++). Two persons gave slight immediate reactions to veal broth A 1:10. One person gave a slight immediate reaction to tetanus media B 1:100, while 2 others gave a similar reaction to the 1:10 dilution of this material. None of these patients showed delayed reactions to either the veal broth A or the tetanus media B. Two gave slight immediate reactions to Berna peptone A, 0.01 mg. of nitrogen per cubic centimeter. One of them also showed a positive delayed response. One showed a moderate immediate as well as a delayed reaction to this peptone. Three persons gave positive delayed reactions though their immediate response was negative. Fifteen gave positive immediate reactions to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. They were recorded as one questionable slight (?), 13 slight, and one marked. Six of these patients also gave positive delayed reactions. One gave a positive delayed reaction to this peptone, although his immediate reaction was negative. Only 2 gave slight immediate reactions to the Difco proteose B, 0.01 mg. of nitrogen per cubic centimeter. Nine persons gave positive immediate reactions to tetanus toxoid A, 1:10; in 3 they were read as questionable slight (?), in 5 they were slight, and in one the reaction was marked. Six of these also gave positive delayed reactions. Three with a doubtful (±) immediate reaction gave a positive delayed response. Six gave a positive immediate reaction to tetanus toxoid B, 1:10. In three the reaction was slight, in 2 it was moderate, and in one it was marked. Three of these also gave positive delayed reactions. One gave a definite delayed reaction though his immediate response to the toxoid B was doubtful (±). The reactions to the finished tetanus bouillon A were in agreement with those obtained with the veal broth A and Berna peptone A.

Three subjects of group 3 were skin-tested thirty-two days after the injection of the second dose of toxoid. In 2 there were no evidences of skin sensitivity. In the third patient only slight positive reactions occurred at the sites of the tetanus toxoid A and B, 1:10, although before the injection of the second dose this person had shown positive reactions to veal broth, Difco proteose, and Berna peptone as well. We cannot account for this discrepancy. In three members of group 4 the skin tests were performed about three to four years after the second dose of toxoid was injected. Patient 19 gave a slight reaction to toxoid B. Patient No. 20 showed slight immediate as well as delayed reactions to tetanus toxoids A and B. Patient 36 gave slight immediate reactions to toxoids A and B and Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. The last two patients were retested thirty-five and twenty-five days, respectively, after a third injection of 1.0 c.c. of alum toxoid was given. Patient 20 then showed slight immediate reactions to toxoids A and B, and a marked reaction to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. Delayed reactions were also present. On the other hand, patient 36 showed immediate marked reactions to both toxoids A and B, and a slight + reaction to the Berna peptone but no delayed reactions.

When skin tests were done two to three years after three to four injections of alum toxoid (varying doses) had been given to the 6 members of group 5, we found that patient 18 showed slight reactions to tetanus toxoids A and B, and that patient 35 showed a slight reaction to toxoid B. The rest of the tests were negative. When retested about one month after the injection of an additional dose of alum toxoid, 3 members of this group, patients 18, 35, and 22, showed slight immediate reactions to tetanus toxoids A and B. Patient 18 also gave a questionable slight reaction to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter and tetanus media B, 1:10. Also patient 30 showed a slight reaction to tetanus toxoid A, while patient 17 gave a similar response to tetanus toxoid B. The most interesting reactions were shown by patient 21. A marked immediate reaction was elicited by tetanus toxoid A and Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter, while a questionable slight response was brought forth by tetanus toxoid B. Veal broth A and Difco proteose B were negative, and tetanus media B gave a slight reaction. Intense delayed reactions occurred at the sites of toxoid A and finished tetanus bouillon A. Similarly, patients 31 and 37 (group 6) showed slight reactions to toxoids A and B. Patient 37 also gave slight reactions to Berna peptone A and veal broth A. After the injection of an additional dose of alum toxoid, patient 31 also showed a slight reaction to Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. Also patients 34 and 38 (group 6) who before the final injection of toxoid had shown negative skin reactions, now gave positive reactions to toxoids A and B. Actually patient 38 showed a moderate reaction to toxoid B. Both of these patients showed delayed reactions. Patient 39 (group 6) remained negative, notwithstanding the four injections of alum toxoid and the three intranasal instillations of toxoid topagen that she had received. Skin tests performed on patients of groups 7 and 8 that received intranasal instillations of toxoid topagen were completely negative except in one. Retests done after the injection of 1.0 c.c. of alum toxoid were also negative. The exception, patient 6, who showed

slight reactions to toxoids A and B, had received an injection of alum toxoid prior to the topagen instillations. The absence of skin reactions in these two groups may be purely accidental, since none of the patients showed any evidence of sensitization even after the injection of 1.0 c.c. of alum toxoid.*

DISCUSSION

Ample evidence is provided by this study in support of the belief that sensitization is an extremely individualized process. Thus, while patient 65 (group 2) gave marked reactions after one single dose of alum toxoid, patient 39 (group 6) showed negative skin reactions after he had received four injections of toxoid and two daily intranasal instillations of toxoid topagen. It is also apparent that sensitization as demonstrated by skin tests occurs rather frequently following the injection of tetanus toxoid alum precipitated.

Particularly worthy of note are the reactions obtained in several patients. Thus patient 25 (group I) showed a slight reaction to tetanus toxoid A, 1:10, and a marked reaction to tetanus toxoid B, 1:10, but was negative to the rest of the test solutions. Positive delayed reactions were produced by both toxoids as well as Berna peptone A, 0.1 mg. of nitrogen per cubic centimeter. These results were confirmed on two subsequent retests. Patient 58 (group 2) showed a slight reaction to Berna peptone A and a moderate reaction to toxoid B, 1:10. The latter also gave a pronounced delayed reaction. The rest of the tests were negative. The same type of reaction to tetanus toxoid B was found in patient 36 (group 4) and in patient 38 (group 6).

These findings would indicate that sensitization to the toxoid protein or to bacterial proteins of *Cl. tetani* has occurred in these patients as a result of the injection of 1.0 c.c. of alum toxoid A. The difference in the skin reaction to toxoids A and B is probably due to differences in concentration of these proteins as found in the two toxoid preparations.

Patient 65 (group 2) gave a marked reaction to toxoid A, 1:10, and Berna peptone A, and a slight reaction to veal broth A and tetanus media B. He was negative to Difco proteose B. Also patient 21 (group 5) showed marked reactions to toxoid A and Berna peptone A, and slight reactions to toxoid B and tetanus media B. These findings would indicate the existence of sensitization to Berna peptone. Confirmatory proof is to be found in the large group of patients that gave slight positive reactions to this protein. It would appear that Berna peptone when present in the alum toxoid used for immunization sensitizes patients more readily than the Difco proteose. This is in agreement with Cooke's observation.

Delayed Reactions.—Patient 29 (group I) is of interest because she gave prominent delayed reactions to toxoids A and B and to Berna peptone A though the immediate reactions to these products were questionable slight positive. The peptone reaction was especially intense. These findings were confirmed on two subsequent rechecks. The mechanism of these delayed reactions is not clear. As expected this patient gave a negative passive transfer test.

*It must be remembered that these patients did not develop any demonstrable antitoxin titer following the intranasal instillation of topagen. The latter is of value only as a repeat stimulus after a basic immunity has been established through the subcutaneous injection of one or preferably two doses of alum-precipitated toxoid.[2]

Similar delayed reactions occurred in a number of our patients. They consisted of either erythema of varying size and intensity or a bright red area of raised induration with a centrally placed papule. Localized swelling of the soft tissues around the site of injection was usually present. Occasionally there was some itching. These delayed reactions began to fade after twenty-four hours and disappeared completely at the end of forty-eight to seventy-two hours. Parish and Oakley[7] also noted "late" reactions "like those occurring after staphylococcus toxoid," but they were at their maximum about two hours after the injection of the testing products and faded completely during the next three hours. Cowles[9] also noted in one immunized person a curious delayed reaction that came on in twenty-four hours.

At present we are unable to evaluate the significance of these delayed reactions. In many cases, an immediate response to the testing solutions occurred as well. The immediate type of reaction was proved by Cooke and associates to be mediated through sensitizing antibodies that circulate freely in the blood and which can be passively transferred to the normal skin (Prausnitz-Küstner phenomenon). We performed passive transfer tests with the serum of patients 21 (group 5), 25, and 29 (group 1) in accordance with the usual technique.[3] Each serum was injected into the skin of three normal nonimmunized persons (0.1 c.c. in each site). Two of the three recipients gave positive reactions at the sites sensitized with the serum of patient 21 when tested with tetanus toxoid B, 1:100, Berna peptone A, 0.10 mg. of nitrogen per cubic centimeter, and tetanus toxoid A, 1:100. All three recipients gave negative reactions at the sites sensitized with the sera of patients 25 and 29.

Duration of Skin Sensitivity.—In the presence of positive skin tests the question arises as to their duration. Cooke reported that one person lost his marked sensitivity within six months. However, another person showed a marked urticarial wheal when retested with tetanus toxoid 1:10 eight months after the second dose had been injected. Passive transfer test with the serum then obtained was weakly positive. We retested patients 21 (group 5), 25, 29 (group 1), 58, and 65 (group 2) six months after the last tests described in the text were performed. At this time patient 21 showed no change in the immediate reactions, but there were no delayed reactions. Patient 25 showed negative immediate reactions to toxoid A and to Berna peptone, 0.10 mg. of nitrogen per cubic centimeter and a moderate reaction to toxoid B, 1:10. Similarly, there was a definite decrease in the size of the delayed responses which occurred at the sites of toxoid A and B and Berna peptone A. Patient 29 showed no change in the reaction to toxoid A, but there was a definite increase in the response to Berna peptone A and to toxoid B. The latter gave a marked reaction. Again the delayed reactions in this patient were very prominent, especially to the Berna peptone. Patients 58 and 65 showed no change in their reactions. On the other hand, the results obtained in the comparative skin tests of several patients suggest that skin sensitization may disappear with time. Thus the absence of positive skin reactions in patient 21 (group 5), when he was tested three years after he had received four injections of alum toxoid as contrasted to the marked reactions obtained one month after the fifth injection of toxoid, can be best explained on the basis that we are dealing here with reactiva-

tion of a sensitivity that had decreased with time. This explanation is more likely to be true than the alternate assumption that the first four injections of toxoid did not sensitize this person while the fifth one did.

Significance of Positive Skin Tests.—It is evident from our studies that a high percentage of persons immunized with tetanus toxoid alum precipitated that contains Berna peptone develop positive skin tests to the toxoid and its component constituents. But as in other fields of allergy, these skin tests to be significant must be correlated and associated with definite evidences of specific clinical sensitivity.

At the outset we were struck by the disparity between the high incidence of positive skin tests and the marked scarcity in our experience of untoward systemic reactions following the injection of two or more doses of tetanus toxoid alum precipitated. A review of the literature reveals a similar paucity of reports of allergic reactions induced by tetanus toxoid. In France where many thousands of men have been injected with three or more doses of Ramon's anatoxin, the procedure was reported as harmless, not being followed by any local or general reaction.[4] In England Boyd[5] also noted the absence of reactions. Recently two very interesting reports have appeared in the *British Medical Journal*. Whittingham[6] reported two cases of anaphylaxis (nonfatal) that occurred among the 61,042 members of the Royal Air Force who had been immunized with two 1.0 c.c. doses of plain toxoid given about six weeks apart. The toxoid contained Witte peptone, which was held responsible for the severe peptone shock, since both patients gave positive skin tests to this protein. Whittingham summarized his experience as follows:

TOTAL NO. PERSONS INOCULATED	ACUTE ANAPHYLACTIC REACTION A	DEFINITE CONSTITUTIONAL SYMPTOMS B	LOCAL REACTIONS ONLY C
61,042	2 patients (0.003%)	12 patients (0.02%)	651 patients (1.06%)

A. Anaphylactic shock.
B. Influenza-like aching of head and body. Slight pyrexia. Seven of these persons had urticaria of limbs and body.
C. Brawny swelling and pain for twelve to twenty-four hours. Twenty-two patients had urticarial swelling and itching around site of inoculation for a few hours.

Parish and Oakley[7] also reported one patient with anaphylaxis after the second injection among over 300 members of the staff of the Wellcome Laboratories that received two injections each of 1 c.c. of plain toxoid. The severe reaction was controlled by epinephrine. This patient had received two courses of staphylococcus toxoid, which has a high content of Witte peptone, two years and one year previous to the administration of the tetanus toxoid. This patient, as well as a number of persons who had received two injections of tetanus toxoid without showing any anaphylactic symptoms, gave marked skin reactions to Witte peptone and to peptic digests of beef, pork, and horse fibrin. Parish and Oakley stated that their observations "throw considerable doubt on the value of scratch and intradermal tests as usually interpreted on the basis of early readings." "Late" reactions appeared to be important. Because the patient in question was the only subject who developed mild anaphylactic symptoms soon after the 1:1,000 dilutions of Witte peptone and two other beef fibrin digests

had been injected intracutaneously, the authors felt that Witte peptone might have been the substance in tetanus toxoid to which the paient had become sensitive. They concluded that allergic phenomena, though alarming, appear to be rare and are readily controlled by the injection of adrenaline. It is important to remember that these two large series of cases were immunized with tetanus toxoid plain, which contains larger amounts of peptone than alum-precipitated toxoids. On the other hand, the latter may act as a better sensitizing agent.

In the United States tetanus toxoid alum precipitated has been used almost exclusively. In their report, Cooke and co-workers refer to two patients with urticaria besides our own case. The two cases were encountered by Hall after the injection of a second dose of toxoid (not alum precipitated). Since publication of Cooke's study, we have immunized 250 additional patients with two 1.0 c.c. doses of alum-precipitated toxoid A without a single systemic reaction. Three persons had severe local reactions which subsided in twenty-four to forty-eight hours. Because individual experiences are bound to be limited in scope, we have collected additional information through the kind cooperation of several workers in this field.[9] This collective experience with tetanus toxoid alum precipitated is summarized in Table I.

Over 1,700 patients have been immunized with two doses of alum toxoid. Many received three injections. Only two cases of urticaria have occurred. One of them resulted after a number of injections had been given to an allergic child for hyperimmunization. The very small number of reactions in this large group of patients is in agreement with the experience of Whittingham[6] and is in sharp contrast with Cooke's "belief that reactions occur more often than the present reports indicate." In his series of about 25 cases, Cooke and collaborators found four instances of induced sensitization with but one reaction of serious proportion. Actually there was only one patient with urticaria, while a second subject gave a very marked swelling with itching at the site of the second injection that subsided in twenty-four hours. In an addendum, Cooke stated that two additional cases of delayed urticaria were encountered. In one it occurred after the second dose; in the other, after the third injection. Neither case showed any significant skin test reaction to the toxoid (1:10) before or after the therapeutic injection.

From the data we have gathered it appears that following immunization with tetanus toxoid alum precipitated skin sensitization occurs much more often than clinical evidences of allergy. Actually the latter are a rather rare occurrence easily controlled by epinephrine hydrochloride. Moreover, an untoward allergic reaction may occur in the absence of any significant skin reaction (Cooke's two cases). Conversely, it does not follow that the presence of a positive skin test contraindicates the administration of tetanus toxoid. To insist upon a negative skin test before the injection of alum toxoid is given is not warranted by our experience. For example, among our own cases after significant positive skin reactions were obtained in two patients (Nos. 58 and 65, group 2), three months after the first dose of alum toxoid A had been given, they received a second dose without any mishap. Six months later these two patients were retested and again after obtaining marked skin reactions to tetanus toxoids A and B and Berna peptone A, a third dose of alum toxoid A was in-

TABLE I

DATA OF REACTIONS IN 1,700 PERSONS AFTER THE INJECTION OF TWO OR MORE DOSES OF TETANUS TOXOID ALUM PRECIPITATED (COLLECTED FROM THE LITERATURE AND PERSONAL COMMUNICATIONS FROM INVESTIGATORS IN THE UNITED STATES)

AUTHOR	NO. OF PATIENTS	NO. OF INJECTIONS	ALUM-PRECIPITATED TOXOID	REACTIONS	SKIN TESTS
A. McBryde*	125 children	2	B and D	No allergic reaction. Occasional moderate severe local reaction	Not done
C. P. Brown*	100	50 had 2 50 had 3	C	None†	Not done
M. Fernan-Nunez*	500	2	D and C	None (remarkedly free of reaction)	Not done
P. B. Cowles*	125	Most had 2 Good many 3	C and B	One student developed about 2° fever with headache and malaise after second injection. Subsided during night	20‡ had skin tests done, with plain tetanus toxoid (bactopeptone) 1:500 saline dilution
R. Layden and W. W. Hall§	199	3	B	None	Not done
H. Gold	500	Most had 2 Many had 3 or more injections	470 had A 10 had B 10 had C 10 had D	One patient had generalized urticaria after second dose. Controlled by adrenaline	96 had skin tests done
A. F. Coca*	180	2	B	118 had local reactions 17 had local and general reactions, the latter consisting of headache, anorexia, malaise, and occasional nausea 5 had only general reaction No allergic reactions were encountered	Not done
R. M. Kempton*	8	2	C	None	Not done

Tetanus toxoid alum precipitated A had Berna peptone.

Tetanus toxoid alum precipitated B had Difco proteose.

Tetanus toxoid alum precipitated C had Martin's peptone.

Tetanus toxoid alum precipitated D had Berna peptone.

*Personal communication.

†A physician reported that an allergic child had a constitutional reaction consisting chiefly of urticaria which occurred after a number of injections had been given for hyperimmunization. (For possible use as blood donor.)

‡Five-hundreths cubic centimeter was injected intracutaneously. Eight weeks after the second injection 3 students gave positive reactions and one a doubtful. The positives were not severe. The most pronounced had an indurated wheal of 1 sq. cm. in an erythematous zone of 4 sq. cm. vanishing in an hour or so. Of these positives one was a curious delayed reaction, similar to the others, but coming on in twenty-four hours. Several tests were made on this person with consistent results. Seven months after these tests, one of the mild reactors received 0.05 ml. of plain toxoid intradermally. Within fifteen minutes there developed an indurated zone about 3.5 cm. in diameter in an erythematous area 7.5 by 15 cm. The wheal persisted for eight hours while the erythema was visible the next day. When the erythema and induration were slight and transient —1 cm.² and lasting only for an hour or so, injections were given without mishap. There was no real reason, other than an excess of caution, for failing to give all reactors the usual recall injection.

jected without producing any allergic reaction. Similarly, two patients (Nos. 25 and 29, group 1) who repeatedly gave very significant positive skin tests of both the immediate and delayed types, received a second injection of alum toxoid without any untoward reaction. These two patients were asthmatic children under treatment.

In the light of this experience, we feel that little value can be attached to the routine performance of skin tests before the injection of tetanus toxoid. The skin test will not materially add to the safety of the injection and will only make cumbersome an otherwise simple and effective method of immunization. On the other hand, if clinical sensitivity to tetanus toxoid does develop and active immunization is required, it is perhaps better to resort to the intranasal route of immunization[2] than to attempt to avoid unpleasant allergic reactions through the fractional multiple dose scheme of injecting the alum toxoid, as recommended by Cooke and others.[1] This study has reaffirmed our previous impression that though allergic reactions may occur after the injection of tetanus toxoid alum precipitated they are rare, and as suggested by Cooke, their incidence can be decreased through the elimination of Berna and Witte Peptone from the medium used for the growth of *Cl. tetani*. Until a synthetic medium is available it would be advisable to use Difco's proteose as a source of peptone. This will not, however, eliminate sensitization to the toxoid protein or the bacterial proteins contained in the immunizing preparation.

SUMMARY

1. In many individuals the injection of tetanus toxoid alum precipitated is followed by the development of a specific sensitivity to the toxoid and some of its constituent proteins, as demonstrated by positive skin tests. Two types of skin reactions are obtained: (a) an immediate reaction (erythema wheal formation) that develops in fifteen minutes, and (b) a delayed reaction which consists of erythema, induration, papule formation, and local swelling that reaches its height in twenty-four hours and then subsides gradually.

2. Berna peptone when present in tetanus toxoid alum precipitated appears to sensitize more readily than Difco proteose. Hence it should be eliminated from the medium in which the tetanus bacillus is grown as suggested by Cooke.

3. Evidence has been presented to indicate that sensitization to the toxoid protein or the bacterial proteins found in the immunizing preparation may also occur following the injection of tetanus toxoid alum precipitated.

4. In our experience there is no apparent correlation between the high incidence of positive skin tests and the low incidence of clinical manifestations of allergy following the injection of alum-precipitated toxoid. This is corroborated by a review of over 1,700 patients immunized with two or more doses of alum toxoid which shows the occurrence of two cases of urticaria following the second injection in one case and a number of injections in the other. This lack of correlation is further demonstrated by the occurrence of urticaria in persons with negative skin tests (Cooke's two cases) and the absence of any allergic reactions following the injection of alum toxoid in four of our patients who had marked positive reactions.

5. Little practical value can be expected from routine skin testing before the injection of tetanus toxoid. This would only serve to render cumbersome an otherwise simple and effective method of immunization against tetanus. As in any other immunizing procedure, the occasional occurrence of an allergic reaction should be kept in mind and epinephrine hydrochoride, 1:1,000, should always be available.

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LOCALIZATION AND CONCENTRATION OF STAPHYLOCOCCUS ANTITOXIN IN AREAS OF RABBIT'S SKIN*

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IN RECENT publications it was shown that staphylococcus antitoxin following an intravenous injection localizes and concentrates in areas of rabbit's skin where xylol is applied.[1, 2] Foreign serum and antibodies have been demonstrated by others in areas of inflammation. Menkin[3] showed the presence of horse serum following an intravenous injection in areas of inflammation by extracting the tissues in saline and using the precipitation reaction. Fox[4] used a similar technique to demonstrate the localization and concentration of agglutinins in areas of inflammation.

The present paper is a report of some observations on the failure of dermal necrosis to occur in the rabbit when staphylococcus toxin is injected intradermally into areas where xylol has been applied in animals previously given staphylococcus antitoxin intravenously.

METHODS AND MATERIAL

Normal rabbits are used. They are carefully shaven twenty-four hours or longer before the experiment is begun. An area, approximately 4 by 6 cm., is outlined with India ink on one side of each rabbit. Five cubic centimeters of staphylococcus antitoxin is given intravenously in the marginal vein of the ear. Xylol, on a cotton applicator, is applied to the demarcated area of skin immediately after the injection of the antitoxin. One tenth cubic centimeter of staphylococcus toxin is injected intradermally in the xylol-treated and untreated skin of the same rabbit at intervals varying from immediately to nine days following the injection of the antitoxin.

In the control experiment xylol is applied similarly to the skin of six rabbits, and staphylococcus toxin is injected intradermally in each of two rabbits at the following intervals after the application of the irritant: five, twenty-four, and forty-eight hours.

The presence or absence of skin necrosis at the site of the injection of the toxin in the xylol-treated areas of skin is considered indicative of either the absence or the presence of antitoxin. The control for each experiment is the presence of necrosis at the site where the toxin is injected in the normal skin.

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A marked variation in the size of the necrosis in each rabbit is also considered significant. Slight variations in the size of the necrosis may be observed when a series of injections of equal size are made in a single rabbit.

TABLE I

NECROSIS PRODUCED BY STAPHYLOCOCCUS TOXIN IN XYLOL-TREATED AND UNTREATED AREAS OF SKIN IN RABBITS GIVEN STAPHYLOCOCCUS ANTITOXIN INTRAVENOUSLY

RABBIT	TIME BETWEEN APPLICATION OF XYLOL AND INJECTION OF TOXIN	AREAS OF NECROSIS AFTER 24 HOURS	
		AREA WITH XYLOL	AREA WITHOUT XYLOL
346	Immediate	0.4 by 0.5 cm.	0.4 by 0.5 cm.
347	Immediate	1.0 by 1.2 cm.	0.5 by 0.6 cm.
399	2 hours	No necrosis	1.5 by 2.0 cm.
400	2 hours	No necrosis	1.0 by 1.5 cm.
363	3 hours, 15 minutes	0.15 by 0.15 cm.	1.2 by 1.2 cm.
364	3 hours, 15 minutes	No necrosis	0.6 by 1.5 cm.
369	24 hours	No necrosis	1.5 by 2.5 cm.
370	24 hours	No necrosis	1.5 by 1.5 cm.
376	44 hours	0.15 by 0.15 cm.	1.0 by 1.5 cm.
377	44 hours	0.2 by 0.2 cm.	1.5 by 2.5 cm.
378	5 days	0.7 by 0.8 cm.	0.8 by 1.4 cm.
379	5 days	0.1 by 0.1 cm.	0.2 by 2.0 cm.
380	9 days	1.5 by 1.5 cm.	1.0 by 2.0 cm.
381	9 days	2.0 by 2.0 cm.	1.5 by 2.0 cm.
367*	5 hours	2.0 by 9.0 cm.	2.5 by 6.0 cm.
371*	5 hours	2.0 by 8.0 cm.	2.0 by 6.0 cm.
393*	24 hours	1.5 by 3.0 cm.	3.0 by 6.0 cm.
391*	24 hours	1.5 by 5.0 cm.	5.0 by 6.0 cm.
388*	48 hours	2.0 by 5.0 cm.	3.0 by 6.5 cm.
389*	48 hours	1.5 by 6.0 cm.	2.5 by 6.0 cm.

*These rabbits received no antitoxin.
Rabbits given 5 c.c. of staphylococcus antitoxin.
All rabbits received 0.1 c.c. of toxin intradermally.

EXPERIMENTAL

The xylol-treated areas of skin become hyperemic and edematous within a period of minutes and continue to show macroscopic evidence of inflammation for approximately five days.

Staphylococcus toxin when injected intradermally produces necrosis in the normal skin within twenty-four hours. Table I shows the size of the necrotic areas in the xylol-treated and untreated skin when staphylococcus toxin is injected intradermally twenty-four hours previously. Furthermore, these data in this table show that all the rabbits in which xylol is applied to the skin and no antitoxin is given intravenously have areas of necrosis in the xylol-treated areas of skin approximately the same size as those in the untreated skin. There is also no significant difference in the size of the areas of necrosis in the treated and untreated areas of skin in those rabbits in which the toxin is injected intradermally immediately following the intravenous injection of the antitoxin.

The areas of necrosis in the normal skin are always smaller in the rabbits given antitoxin than they are in those rabbits given no antitoxin.

When the interval between the intravenous injection of the antitoxin and the intradermal injection of the toxin is two hours, complete inhibition of necrosis occurs in the areas of skin where the xylol is applied. There is also marked inhibition of skin necrosis produced by staphylococcus toxin when

xylol is applied from forty-four hours to five days following the injection of the antitoxin. After nine days the extent of the necrosis produced by the toxin in the area where xylol is applied is the same size as that which occurs in the untreated skin of the same rabbit.

DISCUSSION

The experiment shows that there is an absence of necrosis following an intradermal injection of staphylococcus toxin in those areas of skin where xylol is applied immediately after an intravenous injection of staphylococcus antitoxin when the toxin is given from two to forty-four hours later. This inhibition of local necrosis apparently results primarily from the neutralization of the toxin by the antitoxin. Staphylococcus antitoxin apparently accumulates in a greater quantity in the inflamed tissue than in the normal skin, as shown by the absence of necrosis in the xylol-treated areas. The presence and absence of necrosis in the skin of the same rabbit is a good control and eliminates the necessity of determining the variation in the normal staphylococcus antitoxin titer of each animal.

The size of the skin necrosis produced by staphylococcus toxin in the areas where xylol is applied apparently may be slightly influenced by factors other than antitoxin. This is shown by the slight variation in the size of the areas of necrosis produced by toxin in the rabbits treated with xylol twenty-four to forty-eight hours previously. Some of the staphylococcus toxin may be removed from the site of inoculation in the xylol-treated areas of skin as a result of an increase in the flow of lymph and blood. It is essential to inject the same quantity of toxin into the xylol-treated skin in rabbits given antitoxin as is necessary to produce definite areas of necrosis in the xylol-treated areas of the skin in the same animal without the addition of antitoxin.

It is interesting that an interval of approximately two hours is necessary before a sufficient quantity of antitoxin accumulates where xylol is applied to neutralize completely the amount of toxin used in this experiment. The antitoxic effect, sufficient to inhibit necrosis, remains in the tissue for a limited time only. This is shown by the presence of necrosis where xylol is applied, and the antitoxin is given five days before the intradermal injection of the toxin. Approximately equal size areas of necrosis occur in the xylol-treated and untreated skin when staphylococcus toxin is injected nine days after the xylol is applied locally and the antitoxin is given intravenously.

It would appear most likely that staphylococcus antitoxin localizes and concentrates in areas of tissue as a result of the same mechanism which permits colloidal dyes to localize and to concentrate in areas of inflammation. Staphylococcus antitoxin like trypan blue fails to localize and to concentrate in areas of inflammation when the antitoxin is injected twenty-four hours following the local application of xylol.[1]

The concentration of antibodies in areas of inflammation may produce a local condition unfavorable for the development of an infectious process. Recently such a condition was thought to occur in mice passively immunized with staphylococcus antitoxin when they were given a subcutaneous injection of broth culture of staphylococci. There was a marked increase in the number of

leucocytes in the area of inflammation in the immune animals as compared with the number of leucocytes in the normal.[5]

It is conceivable that antibodies other than antitoxin may localize and concentrate in areas of inflammation provided there is an increase in capillary permeability. The favorable results obtained clinically by applying counter-irritants to local areas of inflammation produced by bacteria may be influenced by the localization of antibodies in the areas as a result of a local increase in capillary permeability.

It may be said that diphtheria antitoxin localizes and concentrates in areas of inflammation produced by the local application of xylol in the rabbit in a manner similar to that of staphylococcus antitoxin. No attempt has been made in these experiments to make a quantitative study on the localization of antitoxin in areas of inflammation.

SUMMARY

Staphylococcus antitoxin following an intravenous injection localizes and concentrates in areas of the rabbit's skin where the capillary permeability is increased through the local application of xylol. This localization of antitoxin is indicated by the development of a smaller area of necrosis where an intradermal injection of staphylococcus toxin is made into the xylol-treated skin. Antitoxin sufficient to inhibit necrosis localizes within approximately two hours and remains in the tissues for at least five days. The significance of the localization of antibodies in areas of inflammation and the use of counterirritants in the treatment of local infections are briefly discussed.

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BACTERIEMIA PRODUCED BY AN AEROBIC, GRAM-NEGATIVE SPORULATING BACILLUS*

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DURING 1938 our laboratory isolated an aerobic gram-negative, spore-forming bacillus from the blood cultures of five hospitalized patients. Because this motile organism on primary isolation apparently did not contain spores or ferment carbohydrates, it was thought at first to be a member of the Alcaligenes group. Further studies, however, revealed its ability to form spores. In addition, it was found to have no serologic relationship to *Alc. faecalis*.

TABLE I

DATA REGARDING ISOLATION OF GRAM-NEGATIVE, SPORULATING BACILLUS

PATIENT	DIAGNOSIS	DATE	BLOOD CULTURE
S	Acute monocytic leucemia	6/27/38	Positive
		7/ 1/38	Positive
		7/ 7/38	Positive
		7/12/38	Negative
		7/20/38	Positive
H	Lymphocytic leucemia and pernicious anemia	7/16/38	Positive
		7/21/38	Negative
		7/23/38	Negative
C	Pneumonitis, tracheobronchial lymphadenitis	8/27/38	Negative
		8/30/38	Negative
		9/ 1/38	Positive
		9/ 8/38	Positive
		9/21/38	Negative
O	Undulant fever	9/14/38	Positive
		9/21/38	Negative
		10/ 7/38	Negative
B	Pneumonia	11/18/38	Positive
		11/22/38	Negative

A total of nine isolations were made over a period of five months (Table I). The blood cultures from three patients were positive once; from one other, twice; and from another, four times. In the last case the gram-negative bacillus was also isolated from the bone marrow and spleen at autopsy nine days after the last blood culture. Morphologically, biochemically, and tinctorially all five strains were identical, and there was but little doubt they comprised a single species. The consistency with which this bacillus was isolated in blood culture from one of these patients convinced us that we were not dealing with an ordinary contaminant, but with an actual inhabitant of the blood stream. Our purpose here is to submit the description of this bacillus and to report the results of serologic and pathogenic studies.

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DESCRIPTION OF ORGANISM

Morphology

Rods: 0.7 to 0.8 by 3.4 to 4.5 microns. Tapered with rounded ends. Occur singly and in pairs, never in chains.

Spores: Ovoid, 0.9 to 1.2 by 1.2 to 1.7 microns. Subterminal to central swelling of the rod, clostridial appearance. Often naked. Formed early (within 18 hours) at 37° C.

Motility: Active and progressive in young cultures. Peritrichous flagella.

Staining: Gram-negative. Young cells sometimes variable.

Cultural Characteristics

Colonies: On extract agar young colonies are medium-sized circular, entire, and regular. They are transparent, colorless, and glossy. Older cultures may contain a variant which is grayish white and opaque. Both types are often present, giving appearance of mixed culture.

Broth: Profuse growth in extract broth with fine pellicle, heavy sediment, and slight turbidity. Streamers often appear in broth.

Biochemical

Carbohydrates: No action on any of the common sugars, or salicin, mannitol, inulin, rhamnose, cellobiose, sorbitol, glycerol, raffinose, inositol, or dextrin. Starch not hydrolyzed.

Gelatin: Liquefaction in from 6 to 8 days at 37° C.

B. C. P. Milk: No coagulation, no acid, becoming alkaline in four to five days. No peptonization in seven days.

Nitrates reduced to nitrites to ammonia; indol negative; slight H_2S production in lead acetate agar. Grows in Koser's uric acid and sodium citrate media. No hemolysis on horse blood agar.

Growth Requirements

Temperature: Minimum temperature for growth, 25° C.; maximum, 50° to 55° C.; optimum, 37° to 42° C.

Optimum pH: Grows at a pH of 5.0 to 9.0. Optimum 6.0 to 8.0.

Inhibition by Dyes: Inhibited by dilutions of gentian violet not ordinarily affecting gram-negative organisms, namely, 1:200,000 to 1:400,000 on extract agar.

Thermal Resistance of Spores: Resists boiling water for 4 hours.

Oxygen Requirements: A strict obligate aerobe.

Antigenic Structure.—High-titered sera were produced by injecting rabbits with young, living, broth cultures. Cross agglutinations with flagellar antigens, as shown in Table II, indicate two serologic types. Strains H and S compose one type, while strains C, O, and B form a second. Reciprocal agglutination tests, using somatic antigens, showed a high degree of cross agglutination and suggested an O antigen common to both types.

TABLE II

RECIPROCAL AGGLUTINATION TESTS

FLAGELLAR ANTIGEN	SERUM DILUTION TITER			
	H	S	C	O
H	5120	10240	160	20
S	5120	10240	160	40
C	320	80	5120	10240
O	320	80	5120	10240
B	160	160	2560	2560

Pathogenicity.—None of the isolations was pathogenic for rabbits. On two different occasions rabbits were injected intravenously with 1.0 c.c. doses of undiluted broth culture on alternate days for a period of two to three months. There were no ill effects. Blood cultures were repeatedly negative. Certain rabbits were sacrificed, and cultures made from the liver, spleen, and kidney. All were negative. Since two isolations (S and H) were from cases of leucemia, daily blood counts were carried out. No significant change in the blood picture was observed.

Comparison With Dubos' Bacillus.—Recently (1939, 1940) Dubos reported the isolation of a bactericidal substance from a soil bacillus (B. G.) which, when first described, appeared to resemble our organism. Morphologically and biochemically they are somewhat similar, but they differ in that Dubos' organism hemolyzes blood and produces spores of low heat resistance. There is no serologic relationship.

One strain of each agglutinative type was fractionated by Dubos' method for obtaining gramicidin. At the same time a search was made for a similar bactericidal substance obtained by Hoogerheide (1940) from members of the *B. subtilis* group. All attempts were unsuccessful.

DISCUSSION

An attempt to classify this gram-negative, spore-bearing bacillus has met with little success. *Bergey's Manual* (1939) contains numerous species of the genus Bacillus which, in many respects, resemble our organism. Nevertheless, of the species described in sufficient detail for accurate comparison, every one is different from the organism being reported in some major morphologic or biochemical characteristic. We believe, however, that an attempt to create a new species would be unwarranted without extensive serologic investigation.

The source and significance of this bacillus is open to conjecture. There is no evidence that it acts as a primary, or even a secondary, incitant of disease. Its presence in the blood stream seemed to bear no relationship to the condition of the patients. No evidence was obtained that the number of organisms in the circulating blood at any one time was high. In addition, of the two sera tested (S and H) neither one contained specific antibodies. The highly resistant nature of the spores suggested that they might be found in saline, or on syringes and needles due to faulty sterilization. In this way, their presence in the blood stream could be explained.

Examination of several lots of glucose solution, saline, and syringes, however, did not yield this organism. The intestinal tract has been considered as a possible source. Nevertheless, cultures from stools of normal individuals were

consistently negative, although from one stool a spore-bearing bacillus of similar morphology was isolated. Each patient whose blood yielded this organism appeared to have lowered resistance. This may account for the survival and possible multiplication in the blood stream of an avirulent spore-former.

Although the literature contains a number of reports of the isolation of *Alc. faecalis* from blood (Andrieu and others, 1936), not all give an adequate description of the organism (Brown and Stewart, 1935; McIntyre, 1936; Goldberg, 1938). It is conceivable that in some of these instances the organism was actually this gram-negative spore-bearer, but due to the absence of spores on primary isolation from the blood stream it was mistaken for *Alc. faecalis*.

SUMMARY

1. An unidentified gram-negative, spore-bearing bacillus was isolated from the blood stream of five different patients.

2. From one individual the organism was isolated four times, from another twice, indicating a state of bacteriemia rather than external contamination.

3. The morphologic, biochemical, and serologic characteristics are presented.

4. The source and significance are discussed.

5. Any relationship to the B. G. organism of Dubos was ruled out. Search for a bactericidal substance by the methods of Dubos and of Hoogerheide was unsuccessful.

The generous assistance of Miss Frances Tuerffs in carrying out the pathogenicity studies is acknowledged with gratitude.

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SEPARATION OF THE COAGULANT FROM THE TOXIC PRINCIPLES OF THE VENOM OF THE AUSTRALIAN TIGER SNAKE (NOTECHIS SCUTATUS)*

With Remarks on the Mode of Action of the Coagulant

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THE venom of the Australian tiger snake *(Notechis scutatus)* is one of the most powerful snake poisons known. Injected subcutaneously, it is more potent per milligram than any other known snake venom.[1] Death is attributable to the action of an extremely active neurotoxin.[2] Tiger snake venom also contains a powerful coagulant substance. Very minute amounts can rapidly clot oxalated or citrated blood in vitro or cause intravascular coagulation in vivo on intravenous injection.[3, 4] Only the venoms of the South American vipers, the jararaca *(Bothrops jararaca)* and the fer-de-lance *(Bothrops atrox)*, and the Australian black snake *(Pseudechis porphyriacus)* are comparable in coagulating power. This property of tiger snake venom has been utilized clinically to arrest severe capillary hemorrhage by topical application.[4] The coagulant action of snake venoms has led also to their introduction into the study of problems concerned in blood coagulability, and has served to increase our knowledge of this process.[5, 6] It has been found that the coagulant factor of tiger snake venom produces its effect in the same way as does thrombokinase, except that the presence of calcium is not required; in other words, it can in itself activate prothrombin to thrombin.[5, 6]

A great deal of work has been done in attempting to separate the coagulant from the neurotoxic factor of tiger snake venom, but hitherto only the toxic factor has been successfully separated.[7, 8] In the present investigation a simple method has been devised to separate the coagulant from the toxic factor of tiger snake venom. It is based on the action of hydrochloric acid upon the venom.

A 1:1,000 solution of powdered tiger snake venom in physiologic salt solution (0.85 per cent NaCl) is made. The solution is filtered through ordinary filter paper to clear it of any debris. Hydrochloric acid, in strength of 1.4 normal or 5 per cent, is added in equal volume. After several minutes an opalescence is noted, which at the end of one-half to one hour forms a precipitate. The resulting mixture is allowed to stand overnight in a refrigerator. The next day the mixture is centrifuged at 2,500 to 3,000 r.p.m. for twenty minutes in order to get a well-packed layer of the precipitate and to effect complete separa-

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tion from the supernatant fluid. The supernatant fluid, which contains the toxin, is carefully decanted or pipetted so as not to disturb any of the sediment in which the clotting factor is present in an inactive state. An extremely small particle of sediment carried over into the supernatant solution will cause the fluid on neutralization to exhibit a significant degree of clotting power. The fluid is, therefore, filtered through a special grade of filter paper (Whatman No. 42), or through a No. 4 Jena fritted glass crucible. The sediment (precipitate) is broken up and dissolved in distilled water. The volume is made up to that of the original venom solution. It is then reprecipitated with 1.4 normal acid. After waiting one-half to one hour the precipitate and the supernatant are separated as before. The processes of precipitation and separation are again repeated. The three precipitations are necessary to free the coagulant present in the sediment from the small amount of absorbed toxin.

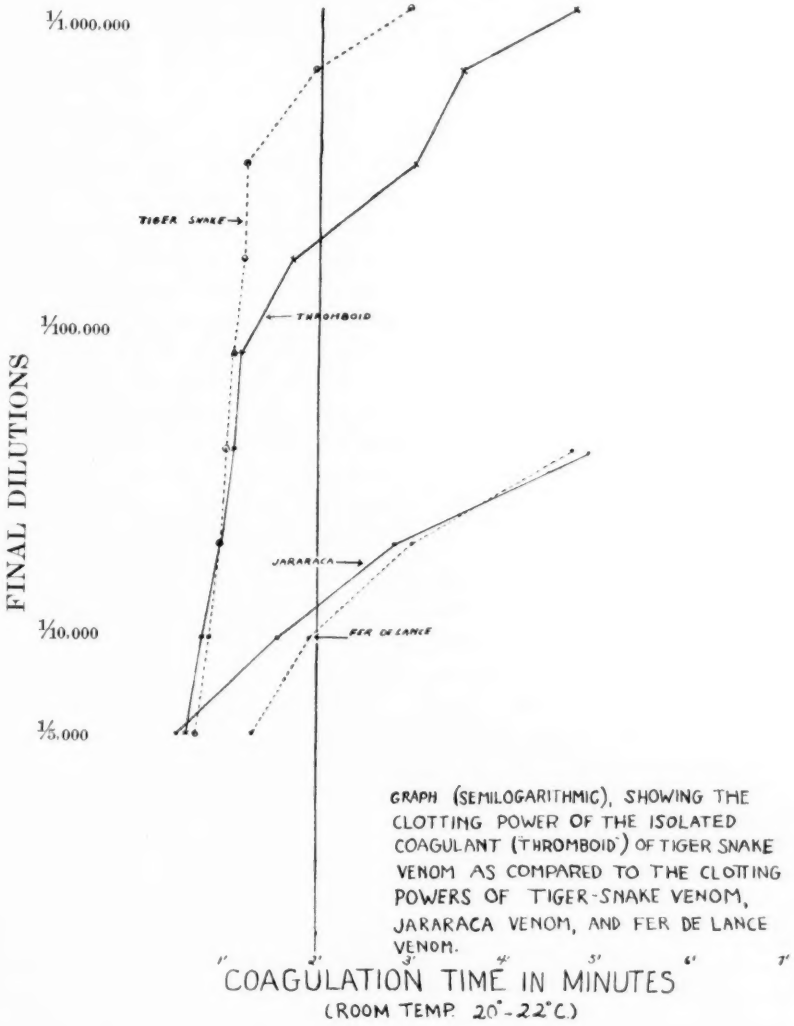
The sediment obtained after the third precipitation is separated by centrifuging as before, and then broken up and dissolved in a volume of distilled water equal to half the volume of the original venom solution. It is now carefully neutralized to a pH of 7.0 to 7.2, or to the pH of the original venom solution. The volume is then brought up to that of the original venom solution with distilled water and is ready for testing. It is essential that the resulting solution shall be water clear. If the solution shows any opalescence it will be much less potent in coagulant activity than a clear solution. During the process of neutralization a heavy precipitation occurs at about a pH 6.5, which may be the isoelectric point of some substance in this preparation. It is important to pass over this point quickly; otherwise a final clear solution may not be obtained.

The three supernatant solutions after filtration are neutralized to pH 7.2, and tested for clotting action. The volumes and any additions are recorded for calculating the dilution of toxin. They are then tested for possible clotting action upon fresh citrated human plasma. Usually no clotting takes place, and the fluids are ready to have their toxicity tested. Occasionally a weak coagulant activity may be noted. When this occurs, the fluids must be treated once with an equal volume of 1.4 normal hydrochloric acid to precipitate the clotting factor, which is then separated as above. The fluids after neutralization are now certain to be coagulant free.

TOXICITY OF THE FRACTIONS

Toxicity tests are made by subcutaneous injections into mice weighing from 15 to 20 Gm. In such animals the average minimal lethal dose of tiger snake venom acting within twenty-four hours is approximately 0.5 c.c. of 1:100,000 solution, or 0.005 mg. It was found that subcutaneous injections of the first supernatant solution, appropriately diluted, killed mice in amounts between 0.75 and 1.0 c.c. of 1:100,000 dilution, which is close to the lethal action of tiger snake venom. The small difference in degree of toxicity between the first supernatant solution and the whole venom solution is mostly accounted for in the toxicity of the second supernatant solution, which was lethal in amounts of 2 c.c. The third supernatant fluid has no significant amount of toxin when injected in doses as large as 3 c.c., the maximal amount of any fluid that can safely be injected at one time in a 15 to 20 Gm. mouse.

In testing the toxicity of the thrice-precipitated fractions (which contain the clotting factor) various preparations were made from amounts of whole venom up to 15 mg., which is equivalent to 3,000 minimal lethal doses in mice. Subcutaneously, inoculated as neutralized solutions in volumes of 2 to 3 c.c., these preparations were found to be harmless. Such mice were observed for a period of one month without their showing any ill effects.



GRAPH (SEMILOGARITHMIC), SHOWING THE CLOTTING POWER OF THE ISOLATED COAGULANT (THROMBOID) OF TIGER SNAKE VENOM AS COMPARED TO THE CLOTTING POWERS OF TIGER-SNAKE VENOM, JARARACA VENOM, AND FER DE LANCE VENOM.

Fig. 1.

THE COAGULANT ACTION OF THE PRECIPITATED FRACTION*

For testing the clotting power of the precipitated fraction, which contains the coagulant factor, a rack of small tubes was set up, each containing 0.1 c.c. of progressive dilutions of the fraction, each tube having one-half the strength of the preceding one. Considering the dilution of this fraction, when the volume

*The term "thromboid" has been applied by us to the precipitated fraction of tiger snake venom solution because of its seemingly thrombinlike action upon citrated plasma without the addition of calcium.

is brought up to that of the original venom solution, to be 1:1,000, the resultant final titers after 0.4 c.c. of citrated human plasma has been added to each tube, ranged from 1:5,000 to 1:1,280,000. Control tubes were set up with similar dilutions of the tiger snake venom from which the fraction was made. It was noted that up to the titer of 1:100,000 the coagulant fraction was just as powerful as the parent substance (tiger snake venom), with clot formation taking place between thirty and ninety seconds, depending on the dilution. In higher dilutions, however, the coagulant fraction rapidly becomes weaker as compared to the whole venom; thus, at a dilution of 1:300,000 the clotting time is twice as long for the fraction as it is for the whole venom. It would, therefore, seem that there is some destructive action by action upon tiger snake venom in the separation of the coagulant factor. It must be borne in mind, however, that the latter was not dried and weighed, and it is reasonable to assume a more powerful action for the coagulant fraction if this were done.

Clotting tests were also made with a purified fibrinogen solution, prepared by triple precipitation with sodium chloride of Berkefeld-filtered citrated plasma.[6] When tiger snake venom or its coagulant fraction is added to the fibrinogen, no coagulation takes place, indicating that these reagents do not act like thrombin. But the addition of prothrombin, prepared by the action of carbon dioxide upon Berkefeld-filtered citrated plasma,[9] enables clotting to take place readily. Hence it appears that tiger snake venom and its coagulant fraction act like thrombokinase, rapidly activating prothrombin into thrombin, without the need of calcium. However, the addition of calcium produces a greater acceleration of the coagulation time. This conception of the thrombokinase-like activity of tiger snake venom and of the coagulant factor derived from it is in agreement with the previous findings of Mellanby[5] and Eagle.[6]

It is interesting to compare the clotting action of tiger snake venom and its derivative coagulant with the potent coagulant venoms of *Bothrops atrox* (fer-de-lance) and *Bothrops jararaca*.* Up to a 1:5,000 titer, jararaca venom has a greater clotting activity than tiger snake venom or its derivative, but then it quickly loses its superiority and at a dilution of 1:10,000 and beyond it is distinctly weaker. The action of fer-de-lance venom closely parallels that of jararaca venom (see Fig. 1). The coagulant activity of still another venom—that of the Russell viper—appears to be far inferior to any of the above-mentioned venoms.

When jararaca or fer-de-lance venom is added to a pure fibrinogen solution, rapid coagulation takes place, indicating that these venoms, unlike tiger snake venom or its derivative, exhibit a thrombin activity.

In comparing the nature of the clots formed by the various venom coagulants, it was found that those produced by tiger snake venom and its derivative are firm and difficult to dislodge, whereas the venoms of the *Bothrops* group produce softer clots, which easily slide down the side of the tube when the latter is inverted. Furthermore, the clots of the *Bothrops* group undergo lysis within twelve hours, while those formed by the tiger snake venom and its derivative do not become fluid until after forty-eight hours.

*For the separation of the coagulant from the toxic factor of the *Bothrops* group of venoms, the method described in this paper is not suitable. For the jararaca venom a method has already been devised.[10]

SUMMARY

A method has been described for completely separating the coagulant from the toxic factor of the venom of the Australian tiger snake *(Notechis scutatus)*. Neither of the fractions was prepared in a dry state, nor was any attempt made to determine its chemistry.

The coagulant fraction is approximately equal in potency to its parent substance up to dilutions of 1:100,000, but in still higher dilutions there is a deterioration in the potency of the fraction. Injected subcutaneously into mice, the coagulant factor prepared from as much as 15 mg. of tiger snake venom, which is equivalent to 3,000 minimal lethal doses, is harmless.

The toxic fraction of the venom not only has no significant coagulant activity but also retains almost quantitatively the toxicity of the original venom.

In its action both tiger snake venom and its coagulant fraction behave like thrombokinase plus calcium, readily converting prothrombin to thrombin. They, therefore, readily clot citrated plasma. Unlike the *Bothrops* venoms, which have a thrombin action, they do not clot purified fibrinogen solutions; however, they will do so when prothrombin is added to the fibrinogen.

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STUDIES ON THE DETECTION OF ABSCESSES AND TUMORS*

III. Concentration and Detection of a Radioactive Substance in Abscesses

H. H. Kroll, B.S., S. F. Strauss, M.D., and H. Necheles, M.D., Ph.D.
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IN AN EARLIER paper a new method for the detection of abscesses or tumors was described. It consisted of the injection of substances which localize in or around abscess or tumor tissue.[1] These substances were made artificially radioactive and their localization was detected by a sensitive electron counter, which registered the points of highest concentration of gamma rays in the body. The blue dye T 1824 was employed. Its concentration in abscessed and normal tissue and various organs was described, and a theory on the mechanism of the localization process, based on physicochemical interpretations, was proposed.[2] Since the purpose of this work was to prepare a radioactive substance, which would localize preferentially in abscess and tumor tissue, and thus be available for diagnostic purposes in man, our attention was turned to other dye derivatives in order to find a more suitable carrier for the radioactive atom.

The various dyestuffs which have been used for studying localization phenomena are mainly vis-azo acid dyes containing three to five sulfonic acid groups. In various studies made on these compounds in order to correlate chemical structure and physiologic activity, the number of sulfonic acid groups in the molecule plays an important role.[3, 4] The most readily available sulfonic acid derivative related to the bis-azo dyes is the disodium 1-amino 8 hydroxy 3,6-disulfonate (H acid), which is used as an intermediate in the preparation of trypan blue and other acid azo dyes. H acid has been found to have an affinity for serum proteins[5] and also to localize in mouse carcinomas.[6]

Because of the ease of bromination, H acid was used in the investigation described in the present paper. It was brominated in aqueous solution, with inactive or with radioactive bromine, and these solutions, after the proper preparation, were injected intravenously into dogs having an abscessed thigh. The localization of the brominated H acid was determined by chemical analysis, while the concentration process of the radioactive compound was followed in the live animal by a Geiger-Mueller counter according to a method described in a previous paper.[1]

Experimental.—Disodium 1-amino 8-hydroxy naphthalene 3,6-disulfonate was obtained from Eastman Kodak Co. The sodium salt was recrystallized from water, and a 1 per cent solution was used. The bromination of the H acid,†[7] injection into the experimental animals, and chemical analysis of the tissues were

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†The bromination of H acid in aqueous solution is accompanied by an oxidation to a 1,4 naphthoquinone derivative.

carried out by methods already described,[2] except that in the present experiments the calculated amount of silver acetate was added to the solution, which was heated to boiling to coagulate the silver bromide, and the solution was centrifuged and filtered to remove the precipitate. All free bromide ions were removed by this procedure.

The radioactive bromine, a mixture of the several possible isotopes, was generated from active lithium bromide by oxidation with potassium bromate. The lithium bromide was activated by exposure to a deuteron beam by means of the cyclotron.

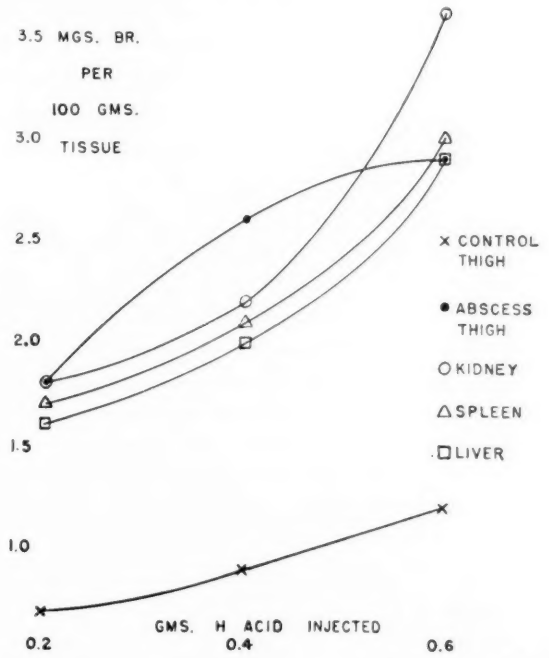

Chart 1.—Distribution of monobrominated H acid in the bodies of dogs.

The calculated amount of lithium bromide and potassium bromate required to monobrominate the H acid was dissolved in 10 to 15 ml. of water and placed in a modified distilling flask connected with a condenser. Three milliliters of 6 N sulfuric acid were added, and the reaction mixture was warmed gently. The bromine was distilled quantitatively into the flask containing the H acid. The bromination proceeded instantaneously, and the reaction was complete when the bromide-bromate solution became colorless, indicating that the lithium bromide had been completely oxidized. The brominated solutions thus prepared were treated in the same manner as the inactive preparations. Extreme care must be exercised to avoid contamination which would produce an increased count in the Geiger-Mueller apparatus.

The localization of the radioactive compound in the experimental animals was determined by the following procedures: An artificial abscess was produced in the thigh as described previously,[1] and twenty-four hours later the animals received an intravenous injection of the preparation; twenty-four hours later they were anesthetized by nembutal, and the following sites of the dog

were focused on the Geiger-Mueller tube: abscessed thigh, normal thigh, lower chest, upper chest, and head. The average total number of counts in a series of three-minute interval readings was used as a measure of the radioactive material concentrated in a given region. The Geiger-Mueller tube is so designed and shielded that the radiations which it may detect must come from the limited area studied. The radiations detected from the radioactive regions of the animal were primarily gamma rays.

TABLE I

CHEMICAL ANALYSIS OF TISSUES AND ORGANS*

SAMPLE	MG. OF Br PER 100 GM. OF WET TISSUE		
	GRAMS OF MONOBROMINATED H ACID INJECTED		
	0.2	0.4	0.6
Abscess thigh	1.80	2.60	2.90
Normal thigh	0.70	0.90	1.20
Kidney	1.80	2.10	3.60
Spleen	1.70	2.20	3.00
Liver	1.60	2.00	2.90

*Animals sacrificed twenty-four hours after injection. The data listed here are the averages obtained from four animals for each concentration studied.

Data.—Table I tabulates the results of the chemical analysis of the tissues and various organs when 0.2, 0.4, and 0.6 Gm. of monobrominated H acid were injected. These data are illustrated graphically in Chart 1. The optimum concentration of monobrominated H acid on the basis of these results is the 0.4 Gm. level. With this dose, the concentration in the abscess was slightly higher or equal to the concentration found in spleen or kidney. At the 0.6 Gm. level the amount of bromine found in the spleen and kidney was greater than that found in the abscess. On the basis of the above results, 0.4 Gm. of H acid was used in the study made with the radioactive compound. The results of the localization of the radioactive H acid are given in Table II.

TABLE II

DISTRIBUTION OF RADIOACTIVE MONOBROMINATED H ACID IN FOUR ANIMALS FOLLOWING INTRAVENOUS INJECTION OF 0.4 GM.

SITE OF COUNT	AVERAGE COUNT PER THREE-MINUTE INTERVAL			
	PREPARATION A	PREPARATION B		
Control*	16	19	22	19
Abscess	57	125	106	105
Normal thigh	31	54	53	51
Lower chest	68	96	133	121
Upper chest	65	109	105	90
Head	54	112	85	64

*Background count due to cosmic and other radiation.

The conclusion drawn from this series of experiments corroborates the chemical analysis. The region of the lower chest, occupied by the kidney and spleen, has a slightly higher concentration of radioactive material than the abscessed thigh in 3 of 4 experiments.

DISCUSSION

The experiments described, although still in the experimental stage, illustrate a new application of the artificially radioactive elements namely, as diag-

nostic indicators for regions of infection. There are many instances where a knowledge of the location of a focus of infection would be of the utmost importance to the surgeon. The preliminary studies made on the location of the brominated H acid in inflamed tissue partly realize this aim, although more experimental work must be done before the use of these indicators may be applied clinically.

The problems to be solved before clinical investigations become possible are (1) the preparation of suitable carriers for the radioactivation; (2) an extensive study of the localization of these carriers in various types of inflammation, tumor growths, and tissue abnormalities; and (3) the technical improvement of the Geiger-Mueller counter so that it may be easily handled and focused on limited areas. It is expected that once the various phases of research listed above are carried to a successful culmination, the radioactive isotope and the Geiger-Mueller counter will prove to be a valuable tool in the hands of the diagnostician.

SUMMARY

The bromination of disodium 1-amino 8 hydroxy naphthaline 3, 6-disulfonate with normal and radioactive bromine is described.

The localization of the inactive brominated derivative in abscess and normal tissue, spleen, kidney and liver, after intravenous injection of varying concentrations, was determined by chemical analysis. The concentration of the radioactive material was determined by locating the regions of highest radioactivity in the live animal by a Geiger-Mueller counter. The application of radioactive isotopes in the diagnosis of tissue abnormalities is discussed.

We want to express our gratitude to Doctors Simon and Slotin from the Department of Chemistry, the University of Chicago, for preparation of the radioactive lithium bromide.

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STUDIES IN ABSORPTION OF UNDIGESTED PROTEIN*

IX. ABSORPTION FROM THE STOMACH AND ESOPHAGUS

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THE absorption of traces of unaltered protein occurs physiologically in fasting adults and children within a relatively short period after oral administration of the protein meal.[1] In studies by Brunner and Walzer[2] fish protein appeared within fifteen minutes after its ingestion in the circulation of 93.8 per cent of the patients tested. In one instance, fish protein was detected in the blood stream two minutes after the consumption of the fish meal. The rapid absorption of other proteins, such as egg,[3] peanut,[4] and cottonseed[5] in an unaltered state, has also been demonstrated. Such absorption occurs regularly, not only following the ingestion of these substances but also following their administration by tube into the duodenum,[6] into the ileum and colon,[7] and into the rectum.[8]

Whether absorption of unaltered protein can occur from the stomach has long been a moot question.[9] In a study of 3 patients with gastric carcinoma with incomplete pyloric obstruction, Gray and Walzer[4] found the absorption rates to be considerably retarded in every instance. This retardation was attributed to mechanical interference with the passage of the ingested protein into the duodenum. Because of the pathologic changes of the gastric mucous membrane in these cases, no conclusions applicable to the normal stomach could be drawn from the investigations.

Further study of the question of gastric absorption was sought by comparing the absorption rates of peanut protein following oral and intraduodenal administration in a series of 14 selected patients.[6] Averages of 24.3 minutes and 18.6 minutes, respectively, were obtained following the oral and intraduodenal administrations of the protein meal. If it could be assumed that no absorption occurred from the stomach or esophagus, the average delay of five or six minutes associated with oral administration might be accounted for as the time needed for the passage of protein from the stomach into the duodenum in amounts sufficient to be absorbed from the small bowel. Previous experimental studies, however, did not eliminate the possibility that absorption of protein might be taking place from the stomach as well as from the small intestine. The need for further information on the subject of absorption from the stomach and esophagus, therefore, became apparent.

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As a result of the pioneer work of Straus it was found that the immunologic technique which had been previously employed to study the absorption of unaltered protein in human beings could be applied effectively to similar studies in the rhesus monkey.[10] This opened up new possibilities for experimental investigation of problems in absorption which had not been feasible in the human being. The present studies were, therefore, undertaken to investigate the question of absorption of unaltered protein from the stomach and esophagus in the rhesus monkey.

TECHNIQUE

The technique employed in these experiments was that described by Straus and Walzer in a previous communication.[10] A cutaneous site on a rhesus monkey was sensitized by the intracutaneous injection of a human serum containing skin-sensitizing antibodies (atopic reagins) for the antigen to be studied. After an interval of from twenty-four to forty-eight hours to permit fixation of the antibodies at the cutaneous site, the specifically related antigen was administered orally. The lighting-up of the sensitized cutaneous site with an urticarial reaction within a few minutes marked the entrance of unaltered protein into the circulation.

The *sensitizing serum* in these experiments was obtained from an asthmatic patient, "P," who manifested marked positive reactions to intracutaneous tests with dilute cottonseed extract. This serum showed an unusually high titer in atopic reagin content for cottonseed protein (1:1,024 by the dilution method of Coca and Grove).[11] To effect local cutaneous sensitization about 0.05 ml. of the undiluted human serum was injected intracutaneously on the thigh or chest of the monkey. An interval of one or two days was then allowed to elapse before the absorption experiments were performed. Food was withheld from the animals for at least twenty-four hours previous to the experiment, but small amounts of water were given. The *protein test meal* consisted of a cottonseed "milk" which was made by mixing 10 Gm. of raw ground cottonseed in 30 ml. of tap water. The method of administration of the test meal and the amount employed varied with the experiment. The *absorption time* was the interval which elapsed between the administration of the test meal and the onset of the reaction at the sensitized cutaneous site.

ABSORPTION OF COTTONSEED PROTEIN FROM THE STOMACH

Experiment I.—This experiment was designed to determine whether or not absorption can occur from the stomach in the rhesus monkey. The animal employed in this study was a female monkey, weighing 4 kg. The details of the experiment are presented in their chronologic order as follows:

November 5, 1937. 9:00 A.M. A cutaneous site on the left chest of the monkey was passively sensitized to cottonseed antigen with an intracutaneous injection of 0.05 ml. of "P" serum.

November 6, 1937. 9:30 A.M. Two grains of veterinary nembutal were administered intravenously. Immediate narcosis resulted.

10:00 A.M. The abdomen was opened. Two clamps were placed at the pyloric end of the stomach. The stomach was completely sectioned between these clamps. The clamps were left in situ.

10:07 A.M. A rubber catheter was introduced into the stomach through the nose. Thirty milliliters of cottonseed "milk" was then injected, through the tube, into the stomach.

10:18 A.M. Wheal formation started to develop at the sensitized cutaneous site, indicating the absorption of the cottonseed protein into the circulation.

The *absorption time* of the cottonseed protein from the stomach in this animal was eleven minutes.

Experiment II.—Before definitely concluding that the positive result obtained in the foregoing experiment was based on absorption of the antigen from the stomach, it was necessary to exclude the esophagus as a possible site of absorption. In this experiment, therefore, the stomach was "isolated" from the esophagus as well as from the duodenum. A male monkey, weighing 3.5 kg., was used in this study.

November 27, 1937. 9:00 A.M. A cutaneous site on the left thigh of the monkey was sensitized to cottonseed with "P" serum.

November 28, 1937. 9:30 A.M. Narcosis was induced by the intravenous injection of 2 grains of nembutal.

9:35 A.M. The abdomen was opened. Two clamps were applied just below the cardio-esophageal orifice, and the stomach was completely sectioned between these two clamps. Just proximal to the pyloroduodenal junction the stomach was completely sectioned between two clamps applied at this site. All clamps were left in situ.

9:56 A.M. Fourteen milliliters of cottonseed "milk" were injected by syringe directly into the lumen of the stomach, through a fine needle penetrating the gastric wall. The stomach was only partially filled by this test meal.

9:58 A.M. Wheal formation started to develop at the sensitized cutaneous site, marking the entrance of cottonseed protein into the circulation.

The *absorption time* of the cottonseed protein from the stomach in this animal was two minutes.

ABSORPTION OF COTTONSEED PROTEIN FROM THE ESOPHAGUS

Experiment III.—This experiment was designed to determine whether or not absorption can occur from the esophagus in the rhesus monkey. For this experiment a male animal, weighing 4.2 kg., was used.

December 3, 1937. 9:00 A.M. A cutaneous site on the right thigh of the animal was passively sensitized to cottonseed with "P" serum.

December 5, 1937. 9:20 A.M. Narcosis was induced with 2 grains of nembutal, injected intravenously.

9:30 A.M. The upper and the lower ends of the esophagus were exposed. The lower end was sectioned between two clamps placed proximal to the cardio-esophageal junction. The upper end was similarly sectioned between two clamps placed approximately one-half inch below the pharyngo-esophageal junction. The clamps were left in situ.

9:56 A.M. The clamp at the upper end of the esophagus was momentarily released, and 7 ml. of cottonseed "milk" were introduced into the esophagus through its exposed upper opening. The clamp was then replaced in its original position.

10:06 A.M. A positive reaction started to develop at the sensitized cutaneous site, indicating the entrance of the cottonseed protein into the circulation.

The *absorption time* of the cottonseed protein from the esophagus in this animal was ten minutes.

DISCUSSION

The experiments were performed on the monkey because these studies obviously could not be done on man. In previous investigations,[10] however, it has been repeatedly demonstrated that the phenomenon of absorption of unaltered protein in the monkey closely simulates that observed in human beings. Moreover, the experimental studies[7, 12] on mucous membrane hypersensitiveness after passive local sensitization have also shown remarkable similarities in both man and the monkey. Under the circumstances, it is reasonable to assume that the absorption of unaltered protein from the "isolated" stomach and "isolated" esophagus observed in the monkey could be demonstrated in human beings under similar experimental conditions.

Attention has been called to the findings revealed in a previous study on human beings that the average absorption time following oral administration of antigen is longer by five or six minutes than the intraduodenal rate.[6] In the light of the present experiments this difference in absorption rate, which might be assumed to be the time needed for the protein to pass from the esophagus and stomach into the duodenum, is now subject to another interpretation. The possibility must be entertained that under certain circumstances the absorption time following oral administration of the antigen may represent the rate of absorption of unaltered antigen from the stomach and even from the esophagus. At any rate, this route of entry of unaltered antigen into the circulation cannot be excluded as a possibility when the antigen is taken orally. In fact, the experimental evidence now at hand indicates that antigens may be absorbed from any part of the alimentary tract, though the rate of absorption may vary somewhat in the different organs.

The amount of antigen necessary to produce the allergic reaction has been shown to be infinitesimally small. In man, Brunner and Baron, in unpublished experiments, have demonstrated that the intravenous injection of cottonseed extract containing 0.0001 mg. of nitrogen is sufficient to induce a reaction at a cutaneous site previously sensitized with the serum used in the present experiments. Using the same serum in experiments on monkeys we, in unpublished experiments, have found that the intravenous injection of cottonseed extract containing 0.0002 mg. of nitrogen was usually sufficient to elicit a reaction at a sensitized cutaneous site. It is obvious, therefore, that only minute amounts of protein need be absorbed into the circulation to produce a reaction at a passively sensitized site. From the point of view of clinical allergy, however, these traces of absorbed unaltered protein may be sufficient to produce severe and even fatal reactions in sensitive patients.

CONCLUSION

The absorption of unaltered protein has been experimentally demonstrated to occur from the esophagus and from the fasting stomach in the rhesus monkey.

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THE IDENTIFICATION OF *BLASTOMYCOIDES HISTOLYTICA* IN THREE INFECTIONS OF THE CENTRAL NERVOUS SYSTEM*

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Synonyms: *Torula histolytica* (Stoddard and Cutler, 1916), *Cryptococcus histolyticus* (Freeman and Weidman, 1923), *Torulopsis histolytica* (Castellani and Jacono, 1933), *Debaryomyces hominis* (Vuillemin-Todd and Herrmann, 1936).

THE presence of *Blastomycoides histolytica*[1] (Stoddard and Cutler, 1916), or a closely allied species, has been reported according to Magruder[2] as the causative agent in 66 cases involving the central nervous system, of which three were found at the University of Virginia Hospital. Recently, Gray[3] added two more cases which occurred in South Africa, and Goldberg added an-

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One other probable unreported case has been found at the Medical College of Virginia. A histopathologic diagnosis of torula meningo-encephalitis was made in 1933 by Dr. Lewis C. Pusch, but was not confirmed bacteriologically. These cases will be reviewed from the neurologic standpoint by Dr. James Asa Shield in a separate paper.

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other one in New York. I wish to report here the bacteriologic and pathologic data on three other cases, bringing the total number* of recorded cases in the literature to 72, and that in Virginia to six.

Since the chaos in the nomenclature of the yeastlike organisms causing these infections has been deplored by Shaw† and others, the synonyms listed above are given. Shaw has pointed out also that not as many species exist as are now recorded and stressed the importance of recognizing their protean manifestations. Further, a review of the literature revealed the fact that seldom has there been a complete bacteriologic description of this yeast accompanying the histologic and clinical studies on the same case, although very adequate cultural or tissue descriptions on separate cases have been made.

CASE 1.—In this case studied by us, *Blastomycoides histolytica* was demonstrated in post-mortem sections and cultures, and was confirmed by mouse experiments as suggested by Crone and associates,[4] in order to standardize a routine procedure for diagnostic purposes. No ante-mortem bacteriologic work was done, although a routine urinalysis showed the presence of yeastlike cells, which were not identified. The clinical diagnosis was that of a brain tumor.

Pathologic Studies:‡ A post-mortem examination was permitted on the head only. No gross pathologic findings suggested torulosis. Microscopic examination of the piarachnoid tissue showed a marked diffuse cellular infiltration. These infiltrationg cells were small round cells and epithelioid-like cells with finely granular eosinophilic cytoplasm and eccentrically placed vesicular lobulated nuclei. There tended to be paravascular granulation tissue nodules composed of the above cell types, both in the meninges and about the vessels within the brain tissue proper. The epithelioid-like cells formed an inner zone about the vessel, while the round cells formed an outer zone. Multinucleated giant cells with eosinophilic cytoplasm containing round clear bodies were found in these nodules. About the vessels in the basal ganglia were "cystlike" areas made of numerous round bodies with doubly refractile borders and a central ground glass substance when stained with hematoxylin and eosin. With Gram's stain these bodies took a deep blue color and were identified as yeastlike cells, probably torula. There was no cellular reaction in these areas. These bodies were also found in the subarachnoid tissue where about them was a marked cellular reaction, focal areas of diffuse hemorrhage, and phagocytosis of some by the above-described multinucleated giant cells (Fig. 1.).

Bacteriologic Studies: A routine culture on blood agar plate of a meningeal swab taken at autopsy revealed, after forty-eight hours, a mucoid, colorless growth. Microscopically, the presence of yeastlike organisms was noted with many budding forms and well-defined cell walls. Transplants on Sabouraud's media and corn meal agar plates showed sufficient growth at room temperature within forty-eight hours for preliminary studies. The growth on corn meal agar always appeared white. On Sabouraud's agar early growth was cream-colored, later becoming yellow, then brown. A mucoid consistency of early colonies was gradually lost. No fermentation of dextrose, lactose, saccharose, maltose, mannite, inosite, arabinose, or xylose occurred. Gelatin was not liquefied. No differences were seen in cultures grown under reduced oxygen tension from those not submitted to this atmosphere. Microscopically, gram-positive, round, and oval budding forms, 4 to 6 microns in diameter, with thick cell walls were observed. No ascospores, capsules, or mycelium were demonstrated (Fig. 2).

Animal Experiments: Six mice were injected intraperitoneally with 0.5 c.c. of saline suspension of a seventy-two-hour culture on Sabouraud's agar at room temperature. The density of the injection suspension was equivalent to standard No. 3 on MacFarland's

*Since the acceptance of this paper for publication, six additional cases[10] of torula infection of the central nervous system have been reported.

†Personal communication from Dr. Frederick W. Shaw.

‡Published with the approval of Dr. Frank L. Apperly, Department of Pathology, Medical College of Virginia, Richmond.

nephelometer. Seven days later an apparently normal mouse was killed. Yeastlike organisms were found in the viscera, lung, heart, brain, and cord as proved by direct smears, cultures, and histologic sections. Grossly and microscopically, no marked changes of the tissues were noted. On direct smears pleomorphism of the organisms (2 to 12 μ diameter) and capsule formations (0.5 to 1.0 μ thickness) were observed, but no ascospores or mycelium. *Blastomycoides histolytica* was recovered in the cultures of the vital organs listed above, with

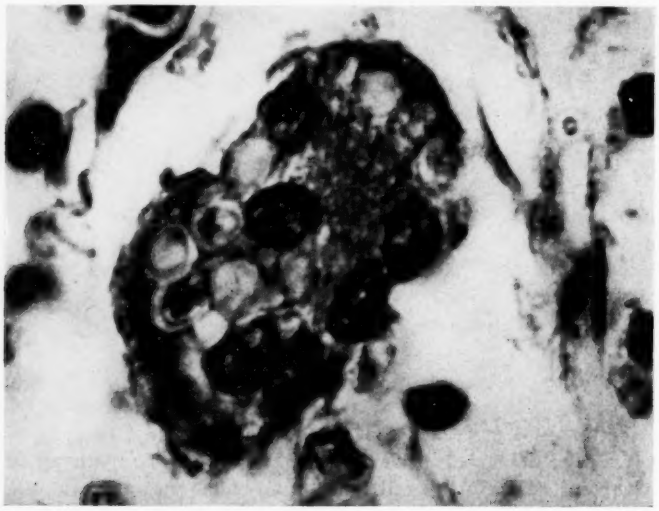

Fig. 1.—Section of human brain. Organisms within giant cell. Hematoxylin and eosin stain (×2,200).

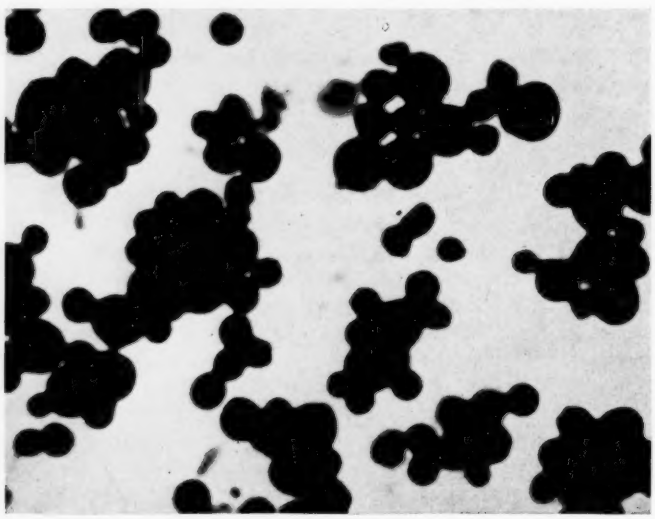

Fig. 2.—Smear preparation of forty-eight-hour culture on Sabouraud's agar from human meninges. Gram's stain (×2,200).

similar growth characteristics of the primary cultures. Four of the remaining inoculated mice died within the following three weeks. The gross and microscopic pathology of yeast meningitis, with a generalized infection characterized by gelatinous tumors with slight inflammatory reactions, marked endothelial hyperplasia, foreign body giant cells, "cystlike" areas, and occasional caseation was seen. *Blastomycoides histolytica* was recovered again by

cultural means. The sixth mouse was killed after four months, although it appeared normal. No gross or microscopic pathology was noted, and no organisms were seen on direct smears. *Blastomycoides histolytica* was recovered, however, in cultures of the lung and liver. Neither agglutinins nor precipitins were demonstrated in the sera of rabbits immunized against antigens prepared as were those of Benham,[5] and Lamb and Lamb[6] (Figs. 3 and 4).

Fig. 3.—Section of mouse brain showing organisms in meningeal exudate. Hematoxylin and eosin stain (×2,200).

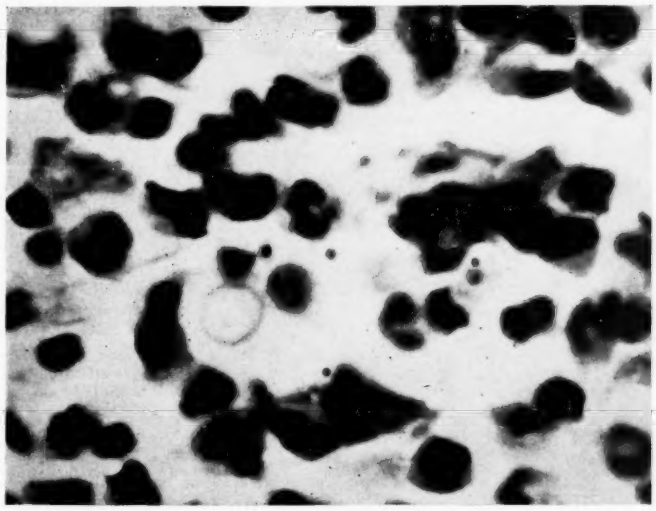

Fig. 4.—Section of mouse lung. Hematoxylin and eosin stain (×2,200).

CASE 2.—This case was diagnosed as meningitis due to yeast infection on March 23, 1938. The patient received specific treatment with iodides and was dismissed. Since then she has remained in apparent good health, except for the blindness which developed during the illness.

Bacteriologic Studies: The first isolation of yeastlike organisms was made from the spinal fluid by cultural means similar to those already given. No animal experiments were run at this time.

Recently upon readmission of this patient to the hospital for observation, the presence of the same yeast was shown in urine and spinal fluid as proved by mouse inoculations with pathologic studies and cultures to be *Blastomycoides histolytica*. Results of the blood, sputum, and stool cultures were negative. No agglutinins or precipitins were demonstrated in the patient's sera.

CASE 3.—Until *Blastomycoides histolytica* was isolated from two specimens of spinal fluid just prior to death, clinically this case was thought to resemble more closely tuberculous meningitis, although no acid-fast bacilli were found on smear preparations or in cultures. Only ante-mortem bacteriologic studies were made, since an autopsy was not granted.

Bacteriologic Studies: The identification of *Blastomycoides histolytica* in two specimens of spinal fluids was accomplished by the same procedures as previously given. In direct microscopic studies no familiar budding forms were seen, although ill-defined lymphocyte-like cells with thick refractile walls were noted in moist preparations and smears stained by Gram's method. The demonstration of acid-fast bacilli was accomplished neither by microscopic examinations nor by cultures on Miraglia's and Petragnani's media. Growth of *Blastomycoides histolytica* was inhibited on Petragnani's media containing malachite green, although it was profuse on Miraglia's at 37° C. Fungous and acid-fast cultures on the urine collected just before death proved negative.

SUMMARY

The increasing number of reports of yeast infections of the central nervous system, especially in Virginia, lead us to believe the condition not to be an uncommon one. Since uncertain clinical diagnoses have been so frequently and generally made upon these cases, emphasis should be placed upon bacteriologic studies prior to death and after it. Many, but not all, stages in the development of *Blastomycoides histolytica*, as pointed out by Todd and Herrmann,[7] were seen. We feel, as do Longmire and Goodwin,[8] that the character of the lesions produced by this yeast is the result of an interrelation of factors, such as the virulence of the strain of organisms, the resistance of the host, and the hypersensitivity of the host. Further studies in cytology and on certain immunologic properties of this organism have been undertaken.

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CLINICAL CHEMISTRY

CHEMICAL STUDIES IN DELIRIUM TREMENS*

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TREATMENT of delirium tremens[1, 2] has consisted mainly of procedures intended to dehydrate the patient. The post-mortem finding of cerebral edema in a large percentage of cases provided the basis for this type of therapy. This regimen has persisted in spite of the fact that many patients are obviously clinically dehydrated.

Attention was called to the unphysiologic nature of dehydration therapy in delirium tremens by Bowman and his co-workers,[3] who showed that dehydrated excited alcoholic persons were benefited by oral or parenteral administration of hypertonic sodium chloride. In a later article,[4] Bowman, Wortis, and Keiser advocated the use of large amounts of fluids, in addition to carbohydrates and sodium chloride, in an effort to readjust metabolism and blood chemistry. On the basis of unpublished data, they stated that the blood chloride and sodium were lowered and lactic acid was increased. The present study was undertaken in an effort to evaluate the physiologic and chemical basis for their recommendations.

MATERIAL AND METHODS

The criteria for the diagnosis of delirium tremens were: (1) History of alcoholism. (2) Visual and auditory hallucinations. (3) Delirium. (4) Excitement. (5) Disorientation. (6) Tremors of the hands and tongue. Patients admitted to the hospital with these findings were studied immediately on admission and after recovery, the latter study serving as control. Treatment varied but in general consisted of sodium chloride, fluids, thiamine, and nicotinic acid administered orally or parenterally. All patients in this series were males.

Blood sugar was determined by the method of Folin-Wu[5]; urea nitrogen by Karr's method[6]; serum proteins colorimetrically by means of the biuret reaction method of Kingsley[7]; and hemoglobin by means of the photoelectric photometer, according to Sheard, Sanford, and Osterberg.[8] Serum chloride was measured by the method of Van Slyke, as modified by Wilson and Ball,[9] total base by the electrodialysis method of Keys,[10] and carbon dioxide combining power by the method of Van Slyke.[11] Spinal fluid proteins were measured by Looney and Walsh's turbidimetric method.[12] Available fluid was determined by the method of Crandall and Anderson[13] utilizing sodium thiocyanate. Hematocrit values were obtained by use of the method described by Peters and Van Slyke.[11]

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TABLE I

DETAILED CHEMISTRY OF PATIENTS STUDIED

"A" and "R" below refer to values on admission and after recovery, respectively.

PATIENT	DATE	BLOOD CHEMISTRY															SPINAL FLUID CHEMISTRY			
		SUGAR (MG. %)	UREA NITROGEN (MG. %)	HEMATOCRIT		HEMOGLOBIN (GM. %)		TOTAL PROTEIN (GM. %)		AVAILABLE FLUID (L./SQ. M.)		CO_2 COMBINING POWER (VOL. %)	CHLORIDES (MEQ./L.)		TOTAL BASE (MEQ./L.)		CHLORIDES (MEQ./L.)	SUGAR (MG. %)	PROTEIN (MG. %)	TOTAL BASE (MEQ./L.)
				A	R	A	R	A	R	A	R		A	R	A	R				A
1	12/ 7/39	70	11	60		16.0		–		–		–	94.0		–		121	55	–	–
2	12/11/39	104	11	–		16.8		6.0		–		45	97.4		–		122	80	68	–
	12/13/39	–	–		–		–		–		–	–		101		–	–	–	–	–
3	12/22/39	126	19	–		14.0		6.8		8.0		48	86.9		–		–	–	–	–
	12/28/39	–	–		39		13.2		7.4		–			99.2		164	–	–	–	–
4	12/11/39	63	9	–		12.7		5.5		–		48	92.3		–		124	60	46	–
	12/18/39	–	–		–		–		–		–	–		101		–	–	–	–	–
5	12/11/39	80	7	–		14.8		4.2		–		66	88.8		–		112	70	32	–
	12/18/39	–	–		–		–		–		–	–		96.5		–	–	–	–	–
6	12/29/39	212	9	42		15.6		8.2		7.4		44	85.0		154		117	127	50	164
	1/ 2/40	123	–		37		13.2		6.5		9.9	–		101		–	–	–	–	–
7	1/ 4/40	84	7	43		14.4		7.4		6.7		38	97.0		–		124	82	38	–
	1/10/40	–	–		41		14.4		9.0		8.8	–		94.8		159	–	–	–	–
8	1/10/40	109	11	43		14.0		7.8		6.6		41	82.8		138		113	89	34	–
9	1/10/40	93	20	50		14.8		8.9		8.9		49	98.4		161		124	67	34	–
	1/18/40	–	–		44		13.6		6.5		8.8	–		94.8		160	–	–	–	–
10	2/ 1/40	75	7	42		14.1		6.4		–		33	97.0		151		125	75	56	–
11	2/ 6/40	132	25	42		10.5		7.6		6.6		39	90.7		155		119	123	56	–
12	7/27/40	96	12	43		14.4		9.4		7.8		40	98.4		155		124	77	50	165
	8/ 1/40	–	–		35		14.0		8.4		9.5	–		107		165	–	–	–	–
13	8/ 1/40	121	13	45		15.2		7.4		8.7		55	89.7		159		119	84	53	162
	8/ 6/40	–	–		42		14.4		7.1		9.1	–		99.0		165	–	–	–	–
14	8/ 2/40	96	9	46		15.4		7.7		6.9		53	92.2		–		124	72	41	159
	8/ 6/40	–	–		36		14.0		6.7		9.1	–		94.8		166	–	–	–	–
15	7/28/40	100	15	41		14.6		6.5		8.9		43	96.5		148		127	89	31	–

RESULTS

On admission serum chloride and carbon dioxide combining power were below normal in nearly all patients. Total base and available fluid determinations showed low or low normal values predominantly. Hemoglobin and serum protein concentrations, together with hematocrit values, were increased. The spinal fluid also showed changes. Chlorides were decreased and protein was increased. On recovery the concentrations of the substances studied returned to normal, with the occasional exception of the serum chloride. In four cases the recovery values for chloride were slightly below normal. The blood sugar and urea nitrogen showed no significant change.

All patients save one recovered; patient 8 died within twenty-four hours after admission as a result of lobar pneumonia. The pneumonia may have contributed to the factors causing the low serum chloride.

TABLE II

MEAN VALUES AND THEIR STANDARD ERRORS OF THE BLOOD CONSTITUENTS

In computing the value for protein, the values for patient 7 were omitted. "A" refers to admission values; "R" to values after recovery.

	HEMATOCRIT		HEMOGLOBIN (GM. %)		PROTEINS (GM. %)		AVAILABLE FLUID (L./M.)		CHLORIDES (MEQ./L.)		TOTAL BASE (MEQ./L.)		CO_2 COMBINING POWER
	A	R	A	R	A	R	A	R	A	R	A	R	A
Mean	45.2	39.1	14.5	13.3	8.1	7.1	7.7	9.2	92.5	99.0	152	163	46
S. E.	±1.5	±1.8	±0.3	±0.5	±0.3	±0.3	±0.3	±0.3	±1.3	±1.6	±2.1	±2.4	

DISCUSSION

The ten patients on whom available fluid studies were performed on admission showed clinical evidence of dehydration. The available fluid values were at or below the accepted lower limits. In six of the ten, available fluid values were less than normal, and in the remainder low normal. After recovery the values were all within normal limits. Comparison of the mean values on admission and after recovery by the method described by Student using Fisher's[14] table of t showed a significant difference. Crandall and Anderson,[13] and Gregerson and Stewart,[15] designate as available fluid, rather than extracellular fluid, the body water determined by the thiocyanate method. Normal values for available fluid are said to lie within the range of 22 to 29 per cent of the body weight, or between 8.7 and 9.2 liters per square meter of body surface area. Crandall and Anderson claim that the estimate of available fluid based on surface area is more accurate and less subject to variation than that based on body weight. This method was used.

Hemoconcentration existed in most, if not all, of the patients on admission. Hemoglobin, protein, and hematocrit values were high on admission when compared with the results of similar studies after recovery. Statistical analysis of the mean values on admission and after recovery showed the differences to be significant.

Total base and chloride determinations revealed hypochloremia and some evidence of base deficiency on admission. In only two of the fifteen patients

studied did chloride fall within normal limits. These two showed figures at the lower limits of normal of 98.0 meq. per liter. In four patients values between 96.0 and 98.0 meq. per liter were observed. All others showed chloride values below 94.0 meq. per liter. Comparison of the mean values of chloride on admission and after recovery indicated the differences to be highly significant. Total base on admission showed a marked departure from normal in only two patients. Recovery was accompanied by an increase in total base in two of the three patients for whom figures are available both before and after treatment. Gojcher and his co-workers[16] noted an increase in serum potassium during chronic alcoholism. In combination with a lowered total base this finding would indicate that the serum sodium may be reduced more than the total base determination shows.

Himwich and co-workers,[17] and Nicholson and Taylor,[18] found an increase in blood lactic acid and a fall in carbon dioxide combining power and in blood pH in acute and chronic alcoholism. The fall in carbon dioxide combining power was confirmed in the present study. It is suggested that a contributory factor in the production of the acidosis in some patients is a deficiency in total base. However, the decrease in total base was less than the decrease in chloride and carbon dioxide combining power, with a resultant rise in undetermined anion. An increase in lactic acid or ketonic acids would provide a plausible explanation. Blood lactic acid, pyruvate, and acetoacetic acid determinations were performed in patient 13. Values only slightly above normal were found. The results, together with chloride, bicarbonate, and protein, left an apparent anion deficit of about 10 meq., suggesting the presence of other organic anions.

Examination of the spinal fluid revealed evidence of altered composition. Disregarding the cases with high blood sugars, about one-half of the remainder showed spinal fluid sugar above normal. A large proportion of the spinal fluid protein values were also above normal. The latter finding suggests either injury to the central nervous system or increased capillary permeability. Low spinal fluid chloride values can be explained by diminution in serum chloride. However, in two cases, in spite of a deficiency in serum chloride, spinal fluid chloride was maintained at normal concentration.

SUMMARY

1. Fifteen patients with delirium tremens were studied chemically on admission and after recovery.

2. Initially hemoconcentration, hypochloremia, lowered alkaline reserve, and some evidence of a decrease in total base were found. The volume of fluid available for solution of thiocyanate was decreased. Clinical and chemical evidence indicated the presence of dehydration.

3. Recovery was accompanied by a restoration of body fluids and electrolytes to normal composition.

4. Delirium tremens should be treated by methods which promote the restoration of fluid and salt.

5. Changes in the cerebrospinal fluid suggested the occurrence of injury to the central nervous system in certain patients.

I wish to express my appreciation to Dr. J. F. Stouffer and the Psychiatric Staff for permission to study their patients, and to Dr. J. G. Reinhold for suggestions and criticism.

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THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF PHOSPHATE SOLUTION IN NORMAL RABBITS

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SHELLING[1] emphasized the importance of phosphates in the successful treatment of animals exposed to the lead hazard and advocated its clinical use. Gray and Greenfield,[2] and later Gant,[3] used the high phosphorus diets clinically in the treatment of patients with industrial plumbism. It was shown experimentally[4, 5] that a high phosphate diet fed to rats during exposure, as well as during deleading, caused a sharp reduction in the amount of lead in the circulating blood. Kowaloff[6] employed a high phosphorus regime in the treatment of a child with lead encephalopathy and showed a marked reduction in the circulating blood lead. In all the cited animal experiments the phosphate was administered orally. The oral administration of any form of therapy in patients with lead encephalopathy may not be practical because of vomiting. Calcium administered to these patients may aggravate the picture since it causes an increase in the circulating blood lead. Under these circumstances, the intravenous administration of a phosphate solution may be a life-saving procedure. Gant[3] employed the alkaline dibasic sodium phosphate solution intravenously in the treatment of plumbism without untoward effects. Sobel[7] and his co-workers have prepared a phosphate solution and used it experimentally in rabbits. They demonstrated that the intravenous administration of their phosphate solution produced a sharp reduction in the blood lead in many experimental animals, in some instances, within one hour after the injection.

The purpose of the following experiments was to observe the behavior of normal animals following the intravenous administration of the phosphate solution prepared by Sobel and his associates. Analyses of the blood and urine were made before and after the injection of the phosphate solution in order to observe the curves of the calcium and phosphorus levels in the blood and in the urine.

The solution used consisted of one part of sodium biphosphate and four parts of sodium phosphate dissolved in 45 parts of distilled water. This made a 10 per cent solution. It was filtered, sterilized, and set up in 10 c.c. ampoules. The pH of the mixture was approximately 7.4 so that no disturbance of the acid-base balance was to be expected following its intravenous administration.

Young chinchilla male rabbits weighing between 2 and 6 pounds were used in these experiments. The animals were anchored on an animal board and catheterized with a No. 6 French ureteral catheter. The marginal veins of both ears were then prepared with alcohol and xylol. After a control specimen of blood was taken from one ear, the phosphate solution was injected into the marginal vein of the opposite ear. The catheter was left in situ, and the urinary drainage was collected in individual containers at hourly intervals so that each

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specimen represented the kidney output for one hour. Samples of blood were obtained from the marginal ear veins at one-hour intervals. Calcium and phosphorus determinations of each of the urine and blood specimens were then made. The animals were kept under observation for four hours. During the experiment the rabbits were fed water by pipette. Food was withheld until after the completion of the experiment.

Experiment 4.—Weight of rabbit 2.95 kg.

The control specimens of urine and of blood were obtained. Five cubic centimeters of the phosphate solution were injected slowly. No abnormal behavior was noted. The specimen of urine obtained at the end of the first hour began to show traces of macroscopic blood. Samples of both urine and venous blood were collected at one-hour intervals for four hours following the completion of the injection.

TABLE I

	SERUM		URINE		
SPECIMEN	CALCIUM (MG./100 C.C.)	PHOSPHORUS (MG./100 C.C.)	VOL. (C.C.)	CALCIUM (MG./100 C.C.)	PHOSPHORUS (MG./100 C.C.)
Control	16.4	4.8	14	134.8	20.2
1st hour	9.4	6.8	15	108.0	98.5
2nd hour	14.4	5.8	14	20.8	48.5
3rd hour	12.9	5.4	9	94.8	33.6
4th hour	12.6	5.3	6	149.2	49.7

Six experiments in all were performed. Table I represents the typical responses noted. In order to determine whether the blood, which appeared following each injection of phosphate solution, was the result of a sudden precipitation of calcium phosphate crystals, two additional experiments were performed. In the first, the catheter was passed into the bladder and left in situ for four hours. No injection was given this animal. Blood appeared in the specimen obtained during the second hour. The catheter was then withdrawn sufficiently to remove the redundant portion of the catheter which, it was felt, was irritating the wall of the bladder. However, enough was left in the bladder to insure a free flow of urine. The subsequent specimens of urine were clear. In the second control rabbit the catheter was passed into the bladder, and after the sphincter reflex was exhausted and the catheter slipped into the bladder, it was withdrawn so that only enough remained in the bladder to insure a free flow of urine. None of the specimens obtained over the period of four hours contained blood. It was, therefore, felt that the blood noted in the specimens obtained in the previous experiments resulted from the irritation to the bladder wall caused by the catheter.

DISCUSSION

All the animals used survived the experiment and were alive for at least six months thereafter. The administration of the phosphate solution to the rabbits caused a sharp rise in the concentration of the serum phosphate as well as in the urine phosphate, and a decrease in the concentration of the serum calcium as well as in the urine calcium. During the second hour there was a secondary rise in the serum and urine calcium levels. The serum and urine calcium values did not return to the control levels within the period during which the animals were kept under observation. It was also noted that the serum and urine phosphate levels showed a secondary decrease, usually beginning within the second hour. Again the recovery fell short of the control level within the

period during which the rabbits were kept under observation. At no time did the observed calcium reach the level which is associated with low calcium tetany. Convulsive seizures were noted in two rabbits and fine tremors in one. These untoward results may have followed the formation of colloidal calcium phosphate, as demonstrated by McLean and Hindriehs[8] in their experimental animals.

Gajato[9] demonstrated that the toxicity of disodium phosphate was three times as great as the toxicity of monosodium phosphate. The pH of the phosphate solution used in the above experiments was 7.4 and closely approximated the pH of the blood. It appears from the experiments that a relatively large amount of this particular phosphate solution (0.463 Gm. per kilogram of body weight) may be administered intravenously. This phosphate mixture does not disturb the acid-base balance as does the disodium phosphate solution when administered intravenously, and, therefore, may be used with greater safety. There are many reports of the intravenous use of phosphate solution in the experimental literature. None have been found in which the phosphate solution was employed as a therapeutic procedure in plumbism. A report of these preliminary experiments was, therefore, considered indicated.

SUMMARY

1. A 10 per cent sodium phosphate solution at the pH of plasma was administered intravenously to rabbits.

2. The serum calcium level dropped and the serum phosphorus level rose following the intravenous administration of the phosphate solution.

3. The concentration of the urine calcium dropped and the urine phosphorus increased following the intravenous administration of the phosphate solution.

4. Convulsions were noted in two animals and tremors in one animal used in this experiment.

5. All rabbits were alive six months following the experiment and appeared to be normal.

I wish to thank Dr. S. M. Gordon, of The Endo Products Company, for his cooperation in preparing this solution suitable for intravenous use, and A. E. Sobel, of the Pediatric Research Laboratory of the Brooklyn Jewish Hospital, for his cooperation and many valuable suggestions.

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LABORATORY METHODS

GENERAL

A NOTE ON THE PROBABILITY OF ERROR IN THE DIAGNOSIS OF RABIES BY MICROSCOPIC SEARCH FOR NEGRI BODIES*

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INTRODUCTION

THE microscopic diagnosis of rabies is accomplished by different workers in various ways; some prefer to look for Negri bodies in paraffin sections, some choose to make impressions, and others make smear preparations. Of the variety of methods the microscopic examination of smears or impressions made from Ammon's horn probably holds pride of place by virtue of its simplicity and the rapidity with which it can be performed. In any case, however, it is probable that a certain number of errors in diagnosis occur due to failure to demonstrate the characteristic intracytoplasmic inclusion bodies in the nerve cells.

Negri-Luzzani[1] reported the results of a study of 4,961 brain specimens by microscopic examination checked by animal inoculation; a total of 3,058 were positive by microscopic examination, but 6.7 per cent of the microscopically negative specimens were also found positive on animal inoculation. Koch and Jahn[2] reported a similar study of specimens received at the Robert Koch Institute during the period 1913 to 1928. Of a total of 8,366 specimens examined, 4,682 were positive, and of those negative microscopically, 11.8 per cent were positive when inoculated into animals. Leach[3] has also reported that of 1,032 brain specimens examined, 12 per cent of those reported microscopically negative were positive on mouse inoculation. Commenting on the probability of such errors in diagnosis, Leach says, "the percentage of error will probably vary also with the prevalence of the disease in the geographic areas from which the material for diagnosis is obtained. In regions of high endemicity one might expect a high percentage of Negri negative specimens proving positive on animal inoculation."

When it is necessary to establish beyond question the diagnosis of rabies, animal inoculation is imperative. Until recently the guinea pig and rabbit have been considered the animals of choice for this purpose, but since the demonstration by Hoyt and Jungeblut[4] that the intracerebral injection of rabies

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virus into white mice produced typical and constant infection, the white mouse has become increasingly popular as a test animal.

The only comparative study of the guinea pig and mouse inoculation methods we have found in the literature is that of Willett and Sulkin.[5] These workers reported that of 25 specimens inoculated in parallel into mice and guinea pigs all were positive in the mice, while four were negative in the pigs. Perhaps this is too small a group to be statistically significant, but it does indicate that occasional positive specimens will be missed by guinea pig inoculation.

EXPERIMENTAL

With a view of testing particularly the accuracy of the foregoing statements as to errors in microscopic examinations, and of determining the actual percentage of error occurring in the routine diagnostic work in our laboratories, the results detailed here are reported. The sources of the material studied were the diagnostic laboratories of the State Department of Health of Alabama during the years 1937 to 1939, inclusive, and of the Georgia Department of Health during 1937.

In the routine procedure the animal heads were received in the laboratories, the brains were removed, and smears were made and examined in the usual fashion. Sellers[6] stain was used to demonstrate the Negri bodies in both laboratories. After the smears had been prepared for direct microscopic examination, the remainder of the Ammon's horn was placed in pure glycerin and sent to the Rabies Laboratory for animal inoculation. Here white mice were injected intracerebrally with 0.03 c.c. of a 1:10 aqueous suspension of the hippocampus after trituration with alundum and centrifugation to throw down coarse particles. Table I gives an analysis of the findings on 1,320 specimens from Alabama, and on 754 specimens from Georgia.

TABLE I

NUMBER OF SPECIMENS AND RESULTS OF MICROSCOPIC EXAMINATION AND MOUSE INOCULATION IN ALABAMA FROM 1937 TO 1939 AND IN GEORGIA DURING 1937

LABORATORY	SPECIMENS EXAMINED	MICRO. − MOUSE −	MICRO. + MOUSE +	MICRO. − MOUSE +	MICRO. + MOUSE −	MICRO. − MOUSE ±	MICRO. ± MOUSE −	MICRO. ± MOUSE +	MICRO. + MOUSE ±
Alabama, 1937	527	353	111	51	5	2	4	1	0
Alabama, 1938	588	377	125	65	6	9	5	0	1
Alabama, 1939	205	167	22	11	0	2	2	0	1
Alabama, Total	1320	897	258	127	11	13	11	1	2
Georgia, 1937	754	445	231	62	6	5	0	4	1

Table I also gives the total number of specimens examined microscopically and subsequently inoculated into mice, together with a complete breakdown of the findings by both methods of diagnosis. Examination of the table brings out two particularly significant points: first, that in both Alabama and Georgia a significant number of specimens are reported as microscopically nega-

tive, which are actually found positive when inoculated into mice; second, that the number of specimens reported positive on the basis of microscopic examination, which are found to be actually negative when injected into mice, is insignificant.

Table II shows the number and per cent of specimens reported microscopically negative but found positive on mouse inoculation, together with the number and per cent of specimens reported microscopically positive but found mouse negative in the Alabama laboratories from 1937 to 1939, inclusive, and in the Georgia laboratories during 1937.

TABLE II

NUMBER AND PER CENT OF SPECIMENS MICROSCOPICALLY NEGATIVE FOUND MOUSE POSITIVE AND THE NUMBER AND PER CENT OF SPECIMENS MICROSCOPICALLY POSITIVE FOUND MOUSE NEGATIVE IN ALABAMA FROM 1937 TO 1939 AND IN GEORGIA DURING 1937

LABORATORY	YEAR	MICRO. NEGATIVE			MICRO. POSITIVE		
		TOTAL SPECIMENS	FOUND MOUSE POS.		TOTAL SPECIMENS	FOUND MOUSE NEG.	
			NUMBER	%		NUMBER	%
Alabama	1937	404	51	12.6	116	5	4.1
Alabama	1938	442	65	14.7	131	6	4.6
Alabama	1939	178	11	6.2	22	0	0.0
Alabama (Total)	1937-39	1024	127	12.4	269	11	4.1
Georgia	1937	507	62	12.2	237	6	2.5

From the figures given in Table II it will be noted that addition of the total microscopically negative specimens and microscopically positive specimens for each year gives a sum total somewhat different from the total number examined for that year, as given in Table I. The reason for this apparent discrepancy is that in Alabama in 1937, for example, there were actually only 404 specimens diagnosed as microscopically negative which when inoculated into mice gave a definite positive or negative result. In other words, the 7 specimens shown in Table I in which either the microscopic or mouse result was not clear-cut, i.e., was doubtful, have been eliminated.

DISCUSSION

It is probable that no single method of microscopic brain examination would yield 100 per cent correct results in the diagnosis of rabies. From the findings presented here it appears that in the laboratories of the Alabama State Department of Health, over a three-year period, slightly over 12 per cent of the specimens reported as negative, on the basis of microscopic examination of stained smears prepared from Ammon's horn, were in fact positive. During 1937 the microscopically negative mouse positive specimens constituted 12.6 per cent of the total diagnosed microscopically negative in Alabama, and 12.2 per cent of the total for the same year in Georgia.

It also appears that errors occur, but to a lesser extent, in the opposite direction. In Alabama, over the three-year period, just about 4 per cent of the specimens diagnosed as microscopically positive were negative when injected into mice. In 1937 in Alabama the number of microscopically positive specimens found negative on inoculation was 4.1 per cent of the total reported micro-

scopically positive, while in Georgia it was 2.5 per cent. However, these figures lose their apparent significance when the total number of specimens examined is considered.

An attempt at explanation of the above contradictions suggests speculation in several different directions: (1) the certainty with which Negri bodies are recognized by the examiners; (2) the failure to examine smear preparations carefully enough or to make them from the portion of the brain in which Negri bodies were present in sufficient numbers to be detected; (3) the presence in the dog population of strains of the virus which fail to elicit Negri body formation in the dog though capable of giving rise to the disease in mice; (4) the prevalence of distemper or other disease in the dog population which might give rise to inclusion bodies that could be mistaken for Negri bodies; (5) the failure to inject enough virus into the mice to cause rabies though Negri bodies were demonstrable in the original smears; (6) the viability of the virus when injected into mice due to its age or other factors.

CONCLUSIONS

1. In an area of high endemicity of rabies errors have been shown to occur in both directions in the microscopic diagnosis of the disease.

2. In Alabama it has been shown that over a three-year period an average of 12.4 per cent of the specimens reported as microscopically negative were actually positive, as shown by mouse inoculation.

3. In Alabama in 1937 the percentage of specimens microscopically negative and found positive on mouse injection was 12.6, while in Georgia the percentage was 12.2 for the same year.

4. In Alabama in 1937 the percentage of microscopically positive specimens found negative on mouse injection was 4.1, while in Georgia it was 2.5.

5. It is not possible to attribute definitely the discrepancies noted above to any one cause.

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A METHOD FOR PREVENTING BLOOD CLOTTING IN CIRCULATION STUDIES*

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SINCE the introduction of methods for measuring blood pressure in acute experiments on laboratory animals, the prevention of coagulation has been an annoying problem. Various methods have been proposed to prevent coagulation, and several anticoagulants have been used, some of which, when introduced into the circulation, are apt to produce toxic manifestations and alter the experimental results. MacCracken and Werness[1] proposed a method in which a portion of the carotid artery was isolated and sectioned, and the proximal end was passed through and everted over the end of a short glass tube and tied in place. It was then placed in a small glass chamber filled with sodium sulfate solution. A side arm of this chamber was connected to a mercury manometer. This device avoided the use of a cannula but was somewhat troublesome to set up, and the authors admitted that clots occasionally formed.

Trendelenburg[2] proposed a method in which the anticoagulant solution (one-sixth molar sodium bicarbonate) was introduced into the cannula in a slow stream. The rate of flow was regulated by dropping mercury into a closed vessel containing the anticoagulant solution and connected to the cannula. This method, while satisfactory in some respects, is open to the objection that the anticoagulant solution is continuously introduced directly into the animal.

In using a mercury manometer marked changes in blood pressure result either in a sudden flow of the anticoagulant solution into the animal or in a complete filling of the cannula with unprotected blood. In the former case the anticoagulant may affect the experimental results, and in the latter case a clot may form in the cannula. Even the pressure changes occurring with the heartbeat continually wash a considerable amount of blood in and out of the cannula, rapidly dissipating the anticoagulant. The use of a membrane manometer† greatly reduces the volume of this ebb and flow.

In an attempt to improve the technique of preventing clotting, a new type of arterial cannula, to be used in conjunction with heparin or other anticoagulants, has been devised. The cannula (Fig. 1), essentially an ordinary three-way wash-out cannula, has a fourth short outlet with a slightly flared rim and is placed at right angles to the other three outlets. A rubber serum vial stopper‡ (*A*) is inserted into the fourth outlet tube, and the thin upper sleeve is inverted over the flared rim of the tube. This securely anchors the stopper. The tip of the cannula is introduced into the artery in the usual manner and is filled with

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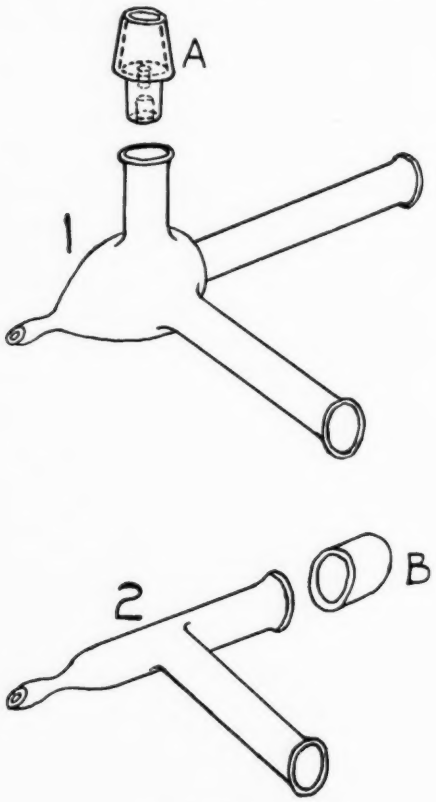
†The Harvard Apparatus Co., Dover, Mass.

‡No. 1A sleeve stopper, obtainable from the West Company, 1117 Shackamaxon Street, Philadelphia.

normal saline under enough force to wash out all air bubbles through the side arm. The side wash-out arm is closed and the connection to the manometer is made. Pressure in the system is raised to about 150 mm. of mercury. The artery clamp is removed and 0.6 mg. of heparin* in 0.15 ml. of solution is introduced immediately through the rubber cap by means of a hypodermic needle (½ inch, No. 25). In blood pressure studies on cats this dose of heparin is usually repeated one to three times during an experiment. Although the solution in the cannula gradually becomes diluted, even with the membrane manometer, the dilution occurs much more slowly than with a mercury manometer.



Figs. 1 and 2.

Any other anticoagulant can be used, of course, but heparin is preferable, since it is the only physiologically normal anticoagulant known and available. Complete heparinization of an animal requires considerably more heparin than three or four of the above doses. According to Reinert and Winterstein,[3] the clotting time of rabbit's blood is longer than one hour, three hours after the intravenous injection of 3,000 a.c.u. per kilogram, or two hours after the injection of 1,500 a.c.u. per kilogram. From this it is estimated that a 3 kg. animal would require fifteen to thirty times as much heparin for general heparinization lasting three hours as for local use by introduction directly into the arterial cannula.

*Heparin was employed in the form of Liquaemin 'Roche' containing 2,000 Roche "anticoagulant units," or 4 mg. per milliliter.

A venous cannula* (Fig. 2) was designed to facilitate intravenous administrations, particularly when a great many injections have to be made during the course of an experiment. The side arm of the cannula is connected to a reservoir of physiologic saline (a 50 c.c. burette is most convenient). The rubber vial cap† (*B*) is slipped over the end of the tube. Injections are made, using a syringe and No. 22 needle, preferably long enough to reach just to the constricted tip of the cannula. The solution is injected and immediately washed in with 1 or 2 c.c. of saline. The rubber cap *B* used with the venous cannula is not satisfactory for the arterial cannula because of the much higher arterial tension, but under venous pressure the cap may be punctured many times without the occurrence of leakage.

SUMMARY

The prevention of clotting during a blood pressure experiment requires a suitable anticoagulant, preferably heparin; a membrane manometer to reduce the volume of the ebb and flow of the blood; and a special arterial cannula herein described for introducing small volumes of the anticoagulant solution.

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*The arterial and venous cannulas were made by the Scientific Glass Apparatus Co., Bloomfield, N. J.

†Size No. 1c gum rubber vial cap also made by the West Company.

THE LAUGHLEN TEST FOR SYPHILIS*

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NUMEROUS reports have been made on the degree of reliability of the Laughlen test. Some have been favorable; others have been adverse.

We wish to report the results of 1,008 consecutive tests on inactivated serum made at the Western State Hospital in comparison with the Kolmer-Wassermann and Kahn tests.

Of the 1,008 specimens, 112 gave reactions of some degree with one or more of the three tests. Most of these patients had syphilis of the central nervous system. Results of 336 tests in these 112 cases were classified as "satisfactory," "questionable," or "unsatisfactory." In the "satisfactory" group all three tests gave positive reactions. Usually, approximately the same strength of reaction occurred with the three tests; however, in many instances a moderate variation occurred. In the "questionable" group a weak reaction occurred with one

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test in the absence of reaction with the other two tests, or there was no reaction with one test in the presence of weak reactions with the other two tests. In the "unsatisfactory" group a strongly positive reaction occurred with one test in the absence of a reaction with the other two tests, or vice versa. It is our purpose not to judge whether, in such instances, the minority or the majority was correct, but simply to compare the tests with each other.

The Kolmer test gave 98 "satisfactory," 8 "questionable," and 6 "unsatisfactory" results. The Kahn test gave 102 "satisfactory," 8 "questionable," and 2 "unsatisfactory" reactions; the Laughlen test gave 106 "satisfactory," 3 "questionable," and 3 "unsatisfactory" results. In two cases in which the Kolmer test was strongly positive in the absence of a reaction to the other two tests, the patients were known to have syphilis; treatment had been instituted. Some of the "unsatisfactory" Kolmer reactions may have been correct, although in the minority.

Judged on the afore-mentioned basis, the Kolmer test showed 99 per cent sensitivity and 91 per cent specificity; the Kahn test showed 98 per cent sensitivity and 93 per cent specificity; the Laughlen test showed 95.5 per cent sensitivity and 99 per cent specificity. "Questionable" reactions were included in computing sensitivity and specificity.

The greater sensitivity of the Kahn test indicates its greater reliability than the Laughlen test as an emergency test for blood donors. If one uses inactivated serum, the Kahn test requires little more time than the Laughlen test.

The Laughlen test appears to be worthy of use with the Kolmer-Wassermann and Kahn tests in providing, without burdening the laboratory unduly, a routine three-test repertoire.

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A PRESSURE SEITZ FILTER FOR LABORATORY USE*

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THERE appears to be a need for a small laboratory sterilizing filter capable of handling small volumes of solutions to render them sterile for injection purposes. The usual laboratory filter is dependent upon suction to draw the solution through it and is, therefore, limited in its use for volatile and viscous solutions. In addition, the suction type actually requires some manipulation to seal the solution into a contamination- and evaporation-proof container under sterile conditions. Many existing filters have a tendency to retain comparatively large amounts of solution and, for this reason, it becomes impractical to use such a filter with small volumes. The device described here is designed to eliminate these difficulties and to make it possible to place the sterilized solution directly into a sterile vial which automatically seals itself against contaminants and evaporation.

Pressure is used to force the solution to be sterilized through a Seitz paper mat. The solution is forced directly into a sterile, rubber-closed vial, thereby eliminating all possibilities of contamination.

The apparatus is made of chromium plated brass and is constructed to withstand a pressure of approximately 150 pounds per square inch. Fig. 1 shows the constructional details. A standard Seitz mat is cut to the desired size and placed over the removable perforated disk *C*. The lower end of the apparatus is tapered to fit a standard hypodermic needle. The two main parts are knurled at *A* and *B* to facilitate the tightening of the Seitz mat. The top plug *D* is fitted with a lead gasket to prevent leakage.

METHOD

The apparatus is assembled, placed in a metal container, and autoclaved for fifteen minutes at 15 pounds' pressure. A vial of an appropriate size to receive the sample and with two hypodermic needles, plugged with cotton and penetrating its rubber cap (see Fig. 2), is autoclaved at the same time. After cooling, one of the cotton plugs is removed, and after flaming the needle, it is attached to the apparatus, and the device is fastened into a support. Since no bacteria can enter the vial, the apparatus may be kept in this condition as long as desired.

Pressure tubing is attached to the air inlet. A high pressure tank of nitrogen or an ordinary compressed air jet may be used to supply the required pressure.

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The sample (1.5 to 10 c.c.) is placed into the apparatus by removing cap *D*. The cap is tightened by means of a rod inserted through its top. Pressure is applied slowly at first, and finally to a point just sufficient to force the liquid into the vial. When the desired sample is obtained, the pressure is released and the needles are removed from the vial.

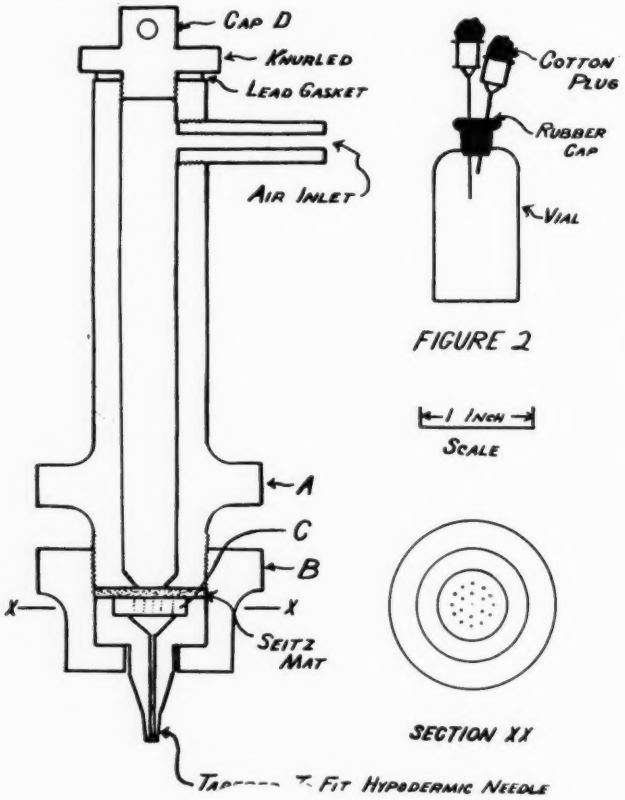

Fig. 1.

The apparatus was tested using a culture of *Bacterium prodigiosum* in nutrient broth. The culture was passed through the filter, and the filter and part of the original culture were plated out. Similar tests were performed using tissue extracts and blood serum. All tests have shown the complete absence of bacteria in solutions passed through the filter.

The apparatus may be secured from the machine shop, of the Department of Biochemistry, the University of Chicago.

MESTER'S TEST IN RHEUMATOID ARTHRITIS AND SPONDYLITIS RHIZOMELIQUE*

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IN 1937 Mester[1] reported results of a salicylic acid skin test in patients with rheumatic and nonrheumatic conditions and concluded that it was a specific diagnostic test for "rheumatic diseases." This conclusion was confirmed in similar studies by Lenoch,[2] and later by Braghin.[3] The latter author reported that the test was positive in all 45 patients who were suffering from rheumatic diseases, including "acute, subacute, and chronic rheumatic polyarthritis, rheumatic ankylopoietic spondylarthritis, rheumatic ischialgia, rheumatic fever with cardiac complications, and chronic rheumatic iridocyclitis." The test was negative in patients with "non-rheumatic" joint diseases, which included gonococcal arthritis, gout, tuberculosis, and syphilis.

If the test were sensitive and specific, as stated by the above-mentioned authors, it would be of great value in the diagnosis of early spondylitis rhizomelique and of atypical atrophic arthritis, and in the exclusion of gout, gonococcal arthritis, and degenerative disease of joints. Since it is sometimes difficult to differentiate these forms of rheumatism, we desired to determine the dependability of Mester's test.

Employing the technique of Braghin,[3] the test was performed in 20 patients with rheumatic disease, 16 of whom had characteristic rheumatoid arthritis, and 4 had spondylitis rhizomelique, and in a control group of 10 nonrheumatic patients. Blood was withdrawn from the middle finger of the right hand of the fasting patient before, and again thirty and sixty minutes after, the administration of two intracutaneous injections of 0.2 c.c. of a sterile 0.1 per cent aqueous solution of salicylic acid, and the number of leucocytes in each blood specimen was determined. The injections were made on the volar surface of the right forearm at a distance of about 5 cm. from each other. These injections were usually followed by pain and burning of short duration, and by the formation, at the sites of injection, of erythematous wheals which disappeared in a few hours. A positive test consists of a transient diminution of the leucocytes, usually within the first thirty minutes, and less frequently within the second thirty minutes after the injection of the salicylic acid solution. A decrease in the leucocyte count of 20 per cent or more was considered a positive test; a rise in the leucocyte count, no change or fall of less than 20 per cent, were considered as negative.

*From the Rackham Arthritis Research Unit and the Department of Internal Medicine, the Medical School of the University of Michigan, Ann Arbor.

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The test gave negative results in all 10 patients with nonrheumatic disease. In the group of 20 with rheumatoid arthritis or spondylitis rhizomelique, a positive test was obtained in only 5 instances (Table I).

RESULTS OF MESTER'S TEST IN PATIENTS WITH RHEUMATOID ARTHRITIS AND SPONDYLITIS RHIZOMELIQUE

DIAGNOSIS	LEUCOCYTE COUNT (CELLS PER C.MM.)			
	BEFORE INJECTION	30 MIN. AFTER INJECTION	60 MIN. AFTER INJECTION	RESULT
Rheumatoid arthritis	10,600	6,100	5,200	Positive
Rheumatoid arthritis	8,850	8,950	9,000	Negative
Rheumatoid arthritis	6,400	6,200	6,200	Negative
Rheumatoid arthritis	7,250	8,100	8,000	Negative
Rheumatoid arthritis	8,800	9,000	8,700	Negative
Rheumatoid arthritis	7,000	7,200	6,900	Negative
Rheumatoid arthritis	7,950	7,700	7,400	Negative
Rheumatoid arthritis	4,100	4,200	4,050	Negative
Rheumatoid arthritis	8,850	5,100	5,400	Positive
Rheumatoid arthritis	4,000	4,200	4,300	Negative
Rheumatoid arthritis	8,100	6,900	8,200	Negative
Rheumatoid arthritis	10,450	10,600	10,400	Negative
Rheumatoid arthritis	6,750	6,200	6,400	Negative
Rheumatoid arthritis	7,200	6,400	6,000	Negative
Rheumatoid arthritis	8,800	8,450	8,400	Negative
Rheumatoid arthritis	8,300	6,150	8,450	Positive
Spondylitis rhizomelique	9,200	9,000	6,100	Positive
Spondylitis rhizomelique	4,550	4,100	4,350	Negative
Spondylitis rhizomelique	11,300	8,400	8,800	Positive
Spondylitis rhizomelique	4,600	4,600	4,450	Negative

Although the test was negative in our control cases, it was also negative in the majority of patients with typical arthritis; in this our results do not agree with those reported by others.[1-3] Braghin, for instance, stated the test was positive in *all* of 45 patients suffering from "rheumatic disease." In our group of 20 patients the test was positive in only 25 per cent. Because of these disappointing results, we did not attempt to determine the "specificity" of the test in differentiating certain rheumatic diseases from gout, gonococcal, and tuberculous arthritis, for even if it were always negative in the latter group, the test would be very unreliable, due to the fact that we found it negative in the majority of patients with characteristic rheumatoid arthritis.

CONCLUSION

Mester's test was found to be grossly unreliable as an aid in the diagnosis of characteristic rheumatoid arthritis and spondylitis rhizomelique, which accordingly makes it of little value in differentiating certain forms of rheumatic disease.

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DETERIORATION OF CORN GERM STEROL*

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IN 1932 Eagle[1] introduced a new sterol, corn germ sterol, for use in fortifying the antigen used in his Eagle flocculation test for syphilis, in addition to the more widely used cholesterol. The major advantage of this sterol as an antigen sensitizer is that the antigen emulsion for use in the test is more stable if corn germ sterol is used than are emulsions made with antigens sensitized with cholesterol alone. In a later modification of the flocculation test[2] the use of both sterols was again recommended.

Recently four preparations of Eagle antigen made in our laboratories from three different lot numbers of Difco beef heart have been found unsatisfactory for use when fortified with old corn germ sterol and fresh cholesterol. These unsatisfactory antigens when tested with sera, which gave a negative reaction to three other nationally recognized tests, show a markedly granular appearance and do not give the "silky swirl" on shaking that is so characteristic in the negative Eagle test. When read microscopically, the granularity observed would justify a reading of doubtful. Strongly positive sera gave characteristic reactions with these antigens, but weak reactions are difficult, if not impossible, to read accurately.

These four unsatisfactory antigens were prepared, one in March, 1940, two in July, 1940, from two different lots of beef heart, and one in August, 1940, using the same beef heart as was used for one of the July preparations. Judging by the degree of granularity observed in tests with negative sera, the deterioration had progressed very rapidly during these five months. This rapidly progressive deterioration is suggestive of an autocatalytic oxidation.

Separate aliquots of three of the four Eagle antigens have been fortified with the same fresh cholesterol and fresh corn germ sterol. With all three of these antigens the negative sera tested have given a characteristic appearance.

The old corn germ sterol was purchased in December, 1932. Difco Laboratories has informed our laboratories that this sterol was made in 1931. Since its purchase this sterol has been kept at room temperature in the brown glass bottle, stoppered with the paper covered cork as received. At this time the old sterol has a yellow tinge and the fresh sterol is white. This color change is also suggestive of a rancidity oxidation. The melting point of this old sterol has been checked at the Difco Laboratories, and it is found to be about 96° C. over a range of approximately 5° C., while in 1931 when the material was prepared the melting point was sharp at 138° C.[3] Eagle antigen prepared in May, 1938, in which this old sterol was used, is still entirely satisfactory.

*From the Division of Preventable Diseases, Minnesota Department of Health.
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From these observations it can be concluded that corn germ sterol deteriorates with age, undergoing an atmospheric oxidation, and is then unsatisfactory for use in Eagle flocculation antigens. It is suggested that this deterioration be guarded against in laboratories in which the Eagle test is used. It seems advisable to keep this sterol in a refrigerator, since this would tend to slow up oxidation. The one sample of sterol here studied was satisfactory for about six years. Other samples might vary in speed of deterioration, and varying laboratory conditions might markedly affect the rate of this chemical change. It, therefore, seems inadvisable to use corn germ sterol for an extended period of time.

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FACTORS INFLUENCING THE DEMONSTRATION OF TUBERCLE BACILLI BY CONCENTRATION METHODS*

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FOR the detection of small numbers of tubercle bacilli in sputum or gastric aspirations the most widely used methods have been culture or animal inoculation. Each of these methods is quite time-consuming, both from the standpoint of the technique employed in the laboratory and the time interval before the clinician receives his report. In addition to this, animal inoculation, which is the more dependable test, is very expensive when used on a large scale. With the increasing use of the laboratory for determining the infectivity of early and convalescent cases of tuberculosis, much study has been expended to determine a method of direct examination which will suffice in a large proportion of such cases and will thus reduce the number of guinea pigs required. A number of methods for concentrating sputum and gastric aspiration have been developed with varying claims for their usefulness. Some investigators have regarded their concentration methods to be superior to guinea pig inoculation. However, it must be remembered that the pathogenicity of an acid-fast organism cannot be determined by the morphology and staining reaction of the organism, and in doubtful cases the guinea pig inoculation is still the final test.

The concentration method depends on (1) the digestion of the mucus and cellular elements in the specimen, and (2) the collection of the organism for staining. The wide variation in the character of the sputum or gastric aspirations leads to difficulty in selecting a digester which will work equally well with all types of specimen. In evaluating the various methods of concentration it

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is necessary to balance the results against the time and expense of procedures. The ideal method to be applied to large numbers of examinations should be simple and should produce a material which is entirely satisfactory for smearing and staining. The concentration should be sufficiently effective so that the material from a large amount of the original specimen can be examined.

The methods for concentrating sputa which are most widely used are the antiformin method of Uhlenhuth and Xylander[7] and the sodium hydroxide method of Petroff.[4] These methods consist of the digesting of the specimen by alkali, centrifuging out the organisms, and making stained smears. To facilitate the separation of the organisms from the digested specimen, flotation with ligroine or other hydrocarbons, as developed by Kinyoun,[3] or precipitation with alum or ferric chloride (Hanks, Clark, and Feldman[2]) have been used. Volatile hydrocarbons were used by Andrus and MacMahon[1] to aid in sedimenting the organisms. Various modifications of these methods have been reported. A number of other types of digesters have more recently been advocated by various workers who attribute to them definite advantages over normal sodium hydroxide. Among these are such vegetable ferments as caroid (Sullivan and Sears[6]), animal ferments as trypsin (Vogt, Zapposodi, and Long[8]), and organic solvents as "tergitol, penetrant 08" (Petroff and Schain[5]). Each of these is more expensive and less available, and their use is more time-consuming than treatment with normal sodium hydroxide. Therefore it would seem that there should be quite an increase in positive results to make them superior to sodium hydroxide for routine use. The same may be said for the various methods for collecting organisms from the digested specimens.

For these reasons, we have made a study of these various methods in comparison with sodium hydroxide. Our chief concern was to find the best method for handling gastric aspirations, but since sputa are more readily available in quantity, the first part of our study was made using sputa, especially mucoid or mucopurulent types, and the methods giving best results were later applied to gastric specimens.

EXPERIMENTAL

The two stages in the concentration of a sputum are: (1) the digestion of mucus and cellular elements, (2) the collection of the organisms for staining.

Digestion.—The technique which we used with sodium hydroxide, and with which we compared other methods, was as follows: To a measured quantity of sputum was added an equal quantity of normal sodium hydroxide and this was shaken well. The mixture was incubated for thirty minutes at 37° C. with occasional shaking. At the end of the incubation period the specimen was shaken well, neutralized to just alkaline to bromthymol blue, and centrifuged for thirty minutes at about 2,000 r.p.m.

Sodium hydroxide has the disadvantage of being injurious to the organisms if left too long in contact with them and may destroy their acid-fast character. It also appears to cause some clumping of the organisms so that distribution on the smear may be irregular. These, and the presence of crystals on the smears, were the disadvantages we tried to overcome.

1. *Alkali.* Other alkalies were tried—calcium hydroxide and ammonium hydroxide—using the same technique. A saturated calcium hydroxide solution

required much longer to be effective, and the results then were not equal to those with normal sodium hydroxide. Normal ammonium hydroxide appeared to liquefy the specimen almost as rapidly as normal sodium hydroxide, but it was in no way superior.

2. *Acids.* Acid digestion with 6 per cent sulfuric acid was tried, using the same technique as above, neutralized with sodium hydroxide and with ammonium hydroxide. This was fairly satisfactory for digesting mucus, although not so rapid in action as normal sodium hydroxide. However, pus cells were left intact, and in mucopurulent specimens the sediment after centrifuging was quite heavy. The smears after this treatment show a nice distribution of the organisms, but sometimes they show extraneous material which is difficult to decolorize.

3. *Animal ferments.* Trypsin is an animal ferment which acts on mucus. However, its action is not sufficiently vigorous to take care of all types of specimens and frequently there is a large volume of sediment or quite a viscous digest.

4. *Vegetable ferments.* Caroid, while it does not digest pus actively, is more satisfactory than trypsin. It appears to be more active in an alkaline medium, hence the addition of equal quantities of a 0.1 to 0.2 per cent sodium hydroxide solution to a 5 or 2 per cent caroid solution is more effective than either would be alone. Occasionally caroid smears are difficult to decolorize, but generally the smears have an even background and good distribution of well-stained organisms. Caroid is less destructive to the organisms than sodium hydroxide, and the digest may be allowed to stand several hours without apparent effect on the staining of the acid-fast organisms.

5. *Organic solvents.* "Tergitol, penetrant 08" in solution with sodium hydroxide, as recommended by Petroff, is an active solvent, liquefying sputum in about the same length of time as normal sodium hydroxide. It appears to be more destructive to the organisms, since fewer organisms were found in the smears from the same specimens after this method of digestion than after digestion with normal sodium hydroxide.

From the study made it appears that the most satisfactory digesters are normal sodium hydroxide and caroid (2 to 5 per cent caroid with 0.2 to 0.1 per cent sodium hydroxide). Normal sodium hydroxide has the disadvantage of being injurious to the organisms, and if left too long in contact will destroy the acid-fast properties. However, there is a wide margin of safety between the time needed for satisfactory liquefaction of the specimen and the time at which injury to the organism is evident. Thirty minutes is sufficient time for liquefaction of all types of specimens except the very tenacious or very purulent. When these types of specimens are mixed with one or two volumes of distilled water, shaken well, and then mixed with an equal volume of normal sodium hydroxide, they, also, are generally liquid in thirty minutes. Reduction in the number of organisms demonstrable by smear is not noticed until more than one hour exposure to normal sodium hydroxide.

Caroid is less destructive to the organisms than sodium hydroxide, and the sediment generally makes very nice smears with well-stained organisms. However, since it does not digest the cellular elements there is frequently a heavy sediment, and sometimes the smears are difficult to decolorize.

Collection of Organisms.—After the specimen is thoroughly liquefied, the organisms are to be collected for staining. In the above studies this was done by centrifugation for thirty minutes at about 2,000 r.p.m. In addition to this, various methods of facilitating sedimentation were compared and a study made of the factors which might influence the separation of the organisms from the digest.

1. To study the effect of specific gravity, aliquots of the digest were centrifuged without dilution, diluted with two volumes of distilled H_2O, and with two volumes of 95 per cent alcohol, respectively. These were centrifuged as above. A comparison of diluted specimens with undiluted specimens gave the following results: Of 20 specimens digested with normal sodium hydroxide, 9 showed more organisms without dilution with water than with dilution, 8 showed more organisms when diluted, and 3 were approximately equal. No definite relationship between type of specimen and the effect of dilution could be discovered. Of 10 specimens diluted with alcohol, 7 showed fewer organisms when diluted, and 3 showed about equal numbers. Since 95 per cent alcohol frequently gives a heavy, rather tough sediment, 50 to 75 per cent alcohol was used instead, or 95 per cent in quantities just short of the amount which would produce heavy precipitation. No definite improvement in number of organisms was obtained.

Of 20 specimens digested with caroid (1, 2, or 5 per cent), 12 showed fewer organisms when diluted with two volumes of distilled water, 6 showed more organisms, and 2 were about equal. Of these same specimens 13 showed fewer organisms when diluted with two volumes of alcohol, 6 showed more, and one was about equal. The above results would indicate that the lowering of the specific gravity by dilution with distilled water or alcohol has no great advantage if the specimen is thoroughly liquefied by the digesting process. However, with specimens which give a viscous digest there appears to be an advantage in dilution. It is more satisfactory to add distilled water (equal volume or more, depending on the degree of viscosity) to the specimen and mix it well before the digester is added. This gives a more satisfactory liquefaction with a lower concentration of the digester; for example, 10 c.c. sputum + 10 c.c. distilled water + 20 c.c. normal sodium hydroxide = a concentration of 2 per cent sodium hydroxide. This gives a more satisfactory digest than 10 c.c. sputum + 20 c.c. normal sodium hydroxide which gives a slightly higher concentration of sodium hydroxide.

2. *Chemical flocculation.* The use of alum, ferric chloride, or sodium carbonate to collect the organisms by flocculation of the proteins was of no definite advantage in our hands. The heavy precipitate from a given quantity of digest greatly reduces the amount of the original specimen which can be used for the examination so that any increase in the proportion of organisms precipitated out is balanced by the smaller quantity examined.

3. *Volatile hydrocarbons.* Following the work of Andrus and MacMahon, specimens, after digestion and neutralization, were shaken with a few drops of chloroform (about 1 drop per cubic centimeter of digest) and centrifuged. The chloroform collects in the bottom of the tube, and the sediment containing the tubercle bacilli forms a layer just above it. This sediment forms a satis-

factory material for staining, giving a good distribution of well-stained organisms. Although the results, again, were not entirely consistent, there appeared to be an advantage in the use of chloroform with digested sputa.

Of 36 specimens digested with sodium hydroxide, 22 showed more organisms when treated with chloroform, 10 showed more organisms untreated, and 4 showed about equal numbers. Of 14 specimens digested with caroid, 7 showed more organisms treated with chloroform, 5 showed fewer, and two showed about equal numbers.

STUDY OF FACTORS INFLUENCING SEDIMENTATION OF TUBERCLE BACILLI

1. *Surface tension depressors.* On the possibility that a lowering of the surface tension of the suspending medium might allow the organisms to be more readily centrifuged, various materials were added to the digested specimen for the purpose of lowering surface tension. These included a stock soap solution, aerosol (0.1 per cent), sodium glycocholate (5 per cent), urea (5 and 20 per cent), and dreft (0.1 and 1 per cent). The results, as with attempts to lower specific gravity, were so varied and inconsistent that no definite conclusions could be drawn. A study of surface tension values was made together with pH values as given below.

2. *Variations in the acids used for neutralizing and a study of pH values.* Sodium hydroxide digests of sputa were used entirely for this part of the work. In the beginning hydrochloric acid was used for neutralization, but since this gave rather heavy salt precipitates the effects of neutralization with sulfuric acid, oxalic acid, and acetic acid were studied. Comparisons were also made of the number of positive slides and the number of organisms per slide found with the sediment from each. The specimens neutralized with acetic acid gave somewhat better results in the number of organisms found and also gave a smear which was easier to examine, showing less extraneous acid-fast material. In the later studies the sodium hydroxide digests were neutralized with 25 per cent acetic acid.

The pH reading and surface tension readings were made on a number of specimens and compared with the count of bacilli on stained smears to determine if there might be a direct relationship between these values and the separation of organisms from the digest. Each specimen was divided into equal parts, and acid was added to secure various degrees of alkalinity or acidity. Other portions of the specimen were treated with alcohol, tergitol, soap, and aerosol to vary the surface tension. Table I shows the pH and surface tension readings of the preparation of each specimen which showed the largest number of bacilli by smear. The smears were made by spreading the material evenly between two slides, and examination consisted of careful observation of three lines across the slide. In some cases more than one preparation gave approximately equal numbers of organisms. In such cases more than one preparation from a specimen is included in the table.

It is noted that there is a wide range of pH and surface tension values through which sedimentation of the organisms goes on equally well. The optimum pH seems to lie between 9 and 11, while the optimum surface tension is between 63 and 73, which is approximately the reading of the neutralized

sodium hydroxide digest without any further treatment. A caroid digest of the average sputum is also in this range. These surface tension readings were made on the Du Nouy apparatus and are based on a reading for distilled water of 108. pH readings were made on the Coleman pH electrometer.

The character of the sputa from patients with tuberculosis or suspected tuberculosis varies to such a degree that we have been unable to lay down a method of examination which is definitely superior for all types of sputum.

TABLE I

SPECIMEN NUMBER	NUMBER OF ORGANISMS	pH	SURFACE TENSION
28	88	12.2	61
29	8	10.0	
	8	11.8	
30	1	5.7	
	1	8.1	
31	1	8.8	63
	1	8.8	67.5
32	4.6 (average per field)	10.15	73
	4.6 (average per field)	9.0	70
	4.3 (average per field)	5.3	61
33	2	9.8	60.5
	2	10.75	75
34	6	11.0	52
35	4	9.0	67
	4	11.1	67
36	156	8.9	73
37	3	9.15	67

The inconsistency of results would suggest that the method of handling is less important than the uneven distribution of organisms in the original specimen. To overcome this we believe that a large quantity of the specimen should be digested. The entire quantity should be digested rather than 5 to 10 c.c., as has been recommended for sputa, or rather than the sediment from centrifuged gastric aspiration. After thorough liquefaction long centrifugation is necessary to sediment the bacilli. The addition of chloroform appears to be an aid in sedimentation, without increasing the amount of sediment. On the other hand, chemical flocculation increases the amount of sediment to such a degree that the sediment from a smaller proportion of sputum is used for the smears and the chance of finding bacilli in the smears is not increased.

Application of Gastric Aspirations.—A total of 287 gastric specimens, which were negative by direct centrifugation were examined by concentration methods. The quantity used varied from 10 to 40 c.c. At first these were digested with equal quantities of normal sodium hydroxide, but it was discovered that 2 per cent sodium hydroxide was satisfactory for these as the proportion of mucus is generally much lower than in sputa. They were digested for thirty minutes at 37° C., neutralized to approximately pH 9 with 25 per cent acetic acid, and centrifuged for thirty minutes at about 2,000 r.p.m. Of the 287, 34 per cent were positive by one method. In 64 specimens an equal portion was shaken with chloroform before centrifugation. Of these 64 specimens examined both with and without chloroform, 25 were positive with chloroform and 23 without chloroform. The advantage of chloroform was less marked here

than in sputa, perhaps because the gastric specimen is more completely liquefied and has a lower specific gravity than the sputum. The small increase in positive results does not appear to warrant the extra handling.

Routine Use in the Laboratory.—For a period of nine months we have been using this method of concentration on routine gastric aspirations and sputum specimens in this laboratory. Letters were sent to the various sanatoriums in the state requesting that, whenever possible, specimens which would ordinarily be sent to this laboratory for guinea pig inoculation should be sent rather for concentration. If this proved negative a second specimen should be sent for guinea pig inoculation. We did not make direct smears as the sanatoriums generally send in only specimens from patients who have been previously negative by smear.

Gastric aspirations were received in the laboratory as either single or pooled specimens (specimens taken on three successive days and pooled) in quantities of from 10 to 100 c.c. The entire quantity was shaken with an equal quantity of 2 per cent sodium hydroxide and incubated at 37° C. for thirty minutes. These were then neutralized with 25 per cent acetic acid to just alkaline to bromthymol blue (pH 8 to 9), and centrifuged at about 2,000 r.p.m. for thirty minutes. In most cases the entire quantity of sediment could be smeared between two slides without making the smears too heavy for examination. These smears were stained by the Ziehl-Neelsen method.

Sputa, unless rather tenacious, were handled in the same way. Those with a large proportion of mucus or pus were first shaken with an equal quantity of distilled water and then with an equal quantity of 2 per cent sodium hydroxide, or if necessary normal sodium hydroxide.

During this period, 807 gastric aspirations and 290 sputa, or a total of 1,097 specimens have been examined. Of these 31.7 per cent of the gastric aspirations and 30.6 per cent of the sputa have been positive by the concentration method; 2.8 per cent of the gastric specimens and 1.3 per cent of the sputa were reported as having atypical acid-fast bacilli present.

One hundred and fifty-four specimens from patients who had received negative reports by concentration on previous specimens were injected into guinea pigs. More than half of these were pooled specimens. Two guinea pigs were injected in such cases. Of the 154 specimens, 89 per cent were negative by guinea pig inoculation and 11 per cent were positive. Of this 11 per cent (17 specimens), 4.5 per cent (7 specimens) were positive in one guinea pig and negative in the other, indicating that the organisms were very few in number. We believe that the positive results gained by careful examination of the concentrated specimens more than compensates for the time required for preparing the specimens and examining the slides, since tubercle bacilli can be demonstrated immediately in many specimens which would otherwise require guinea pig inoculation and a delay of six weeks before the bacilli could be demonstrated.

CONCLUSIONS

1. The wide variation in results obtained through the use of various methods of digesting sputa and gastric specimens and precipitating the tubercle bacilli from the digests indicates that the newer methods of handling have lit-

tle, if any, advantage over digestion with normal sodium hydroxide (2 per cent sodium hydroxide with gastric specimens or less mucoid sputa) and long centrifugation.

2. Comparable results in precipitating the organisms can be obtained through a fairly wide range of pH values (9 to 11) and surface tension readings (63 to 73).

3. Dilution with distilled water or alcohol to lower specific gravity was of no value except in the case of very viscous specimens. In such cases dilution with distilled water before digestion aids in liquefaction, and it is not necessary to increase the concentration of the digester.

4. The use of a large amount of specimen, thorough liquefaction and long centrifugation, thus securing a great concentration of the solids, are the essentials of a good concentration method.

We would like to express our appreciation to Dr. W. H. Oatway, Jr., of the Wisconsin General Hospital and to the physicians of various sanatoriums in the state for furnishing us with specimens.

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A BLOOD LANCET ASSEMBLED FROM COMMON LABORATORY ACCESSORIES*

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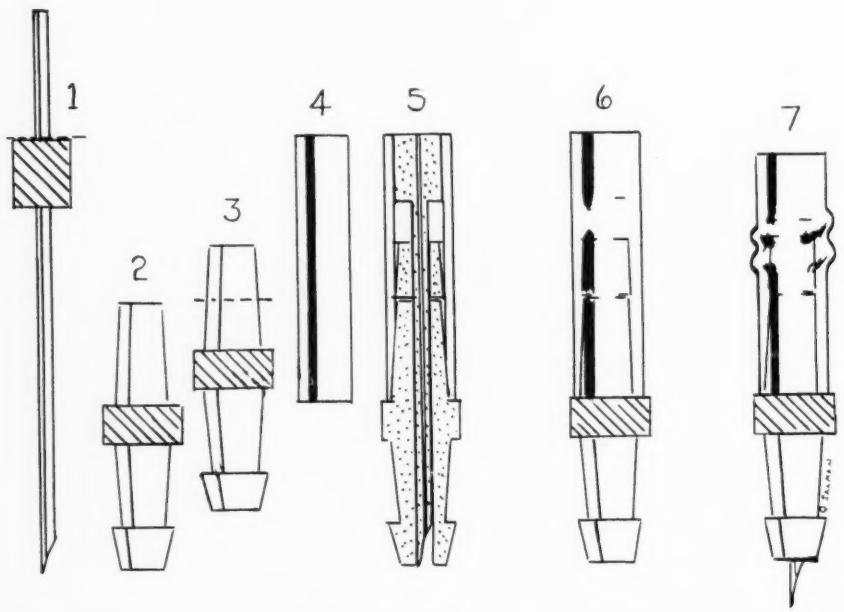
BLOOD lancets, because of their size and constant use, are easily lost or misplaced. Internes on laboratory service are notorious for losing them. Consequently, the usual substitute for a lancet is an ordinary needle stuck through a cork. Even though such instruments are effective, it must be granted that the depth to which the needle will penetrate is not readily controllable. In addition, such an instrument often shocks the finer sensibilities of many patients. Certainly, it does not look like an instrument of precision which is expected of the medical profession.



Figs. 1-7.—*1*, New York Board of Health bleeding needle, gauge No. 19. *2*, Needle adapter for ends of rubber tubing. *3*, A second adapter to be cut at the dotted line. *4*, Rubber tubing as employed for blood counting pipettes. *5*, Cross section of the assembled parts. *6*, Normal appearance of the instrument. *7*, Shows the needle point protruded beyond the shield during action.

A practical lancet can be easily assembled from the following parts: An ordinary blood-letting needle of the New York Board of Health Type, No. 19 gauge, and 1¼ inches long (Fig. 1); two needle adapters for ends of rubber tubing (Fig. 2 and Fig. 3); and a short piece of rubber tubing of the size used

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on blood counting pipettes (Fig. 4). The blunt end of the needle (Fig. 1) and one of the adapters (Fig. 3) are cut off at the dotted lines. All parts are assembled and held together by the rubber tubing, as shown in Fig. 5. This figure shows the lancet in cross section with the needle in its normally retracted position. Fig. 6 shows the completed instrument, while Fig. 7 demonstrates the projection of the needle point. The rubber tubing serves a double purpose of holding the parts together and of acting as a spring for the retraction of the needle point.

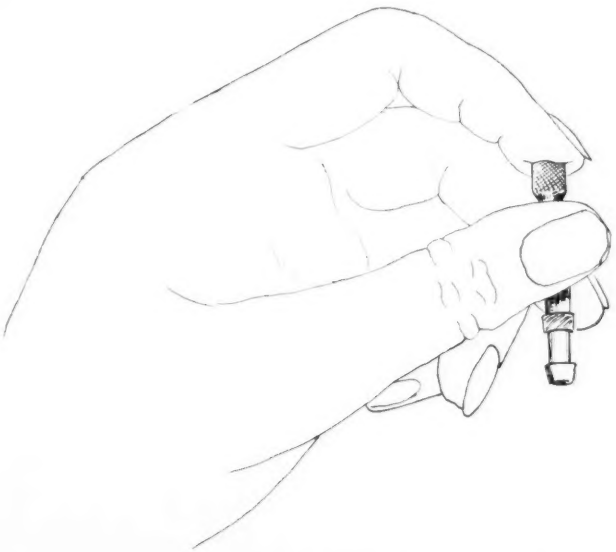

Fig. 8.—The comparative size and position of the instrument.

No dimensions are given, since all parts are of standard size. The only variable is the small section of the adapter in Fig. 3. The length of this section depends on the length of the needle available, so that the latter may extend beyond its normal position to the desired distance when it is penetrating the patient's skin. Fig. 8 shows the relative size of the finished instrument.

The lancet is used by holding it between the thumb and middle finger, as shown in Fig. 8. While one end is resting on the patient's skin, the other is given a quick tap with the index finger. This maneuver will propel the needle beyond its shield to the predetermined depth. The resiliency of the rubber tubing will return the needle to its former protected position.

The lancet has been used for some time and has proved entirely satisfactory.

A MICROMETHOD OF THE WELTMANN REACTION*

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THE serum coagulation test, as introduced by Weltmann[1-3] in 1930, is a valuable aid for the differentiation between exudative, inflammatory, or necrotic processes, and fibrotic, proliferative conditions. The value of this reaction was confirmed by many German investigators.[4-8]

Recently, in this country, Levinson and associates[9] carried out a study of the Weltmann reaction on a large series of various diseases as pneumonia, pleuritis, cirrhosis of the liver, sepsis, and especially endocarditis. Their findings confirmed the earlier reports of the worth of this test.

In a second paper Levinson and Klein[10] demonstrated the importance of this test in distinguishing between exudative and proliferative types of lung tuberculosis. They say: "in tuberculosis the Weltmann coagulation test is of importance, because it can be used as a guide in the course of, and the prognosis of the disease. It reflects the tissue changes in the body." However, they write: "When exudative and proliferative changes occur in the lungs at the same time, as happens frequently in tuberculosis, the Weltmann reaction may not be of great assistance. The two divergent activities occurring at the same time result in the coagulation band, striking the normal zone." Although, the standard technique is described in the above-mentioned articles,[9, 10] we shall repeat the description of the original method.

A 10 per cent stock solution of calcium chloride ($CaCl_2$. $6H_2O$) is prepared. From this solution various dilutions are made, ranging from 0.1 per cent to 0.01 per cent. These solutions are numbered from 1 to 10, beginning with the strongest concentration. Small test tubes, similar to those used for the Wassermann reaction, are placed in a metal rack. These tubes are also numbered from 1 to 10. Five cubic centimeters of the similarly numbered calcium chloride solution are placed in each test tube. To each tube is added 0.1 c.c. of unhemolyzed serum. The solutions are thoroughly mixed, and the tubes are placed in a hot water bath and boiled for fifteen minutes, after which time the tubes are examined. The tube with the solution of highest dilution, at which flocculation is present, is noted. Weltmann called the concentrations at which flocculation occurs as the coagulation band, and designated it as K.B. Normal K.B. is given to the reaction if there is flocculation up to and including the sixth tube. Other workers included the seventh tube as normal.

The findings of various investigators reveal that exudative and necrotic processes as pneumonia, pleuritis, caseous type of lung tuberculosis, recent coro-

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nary thrombosis, lung abscess, and other such conditions cause a narrowing of the K.B. (a shift to the left), or in very severe cases, an absence of any coagulation.

TABLE I

COMPARISON OF THE CONCENTRATIONS BETWEEN THE ORIGINAL METHOD AND THE MICROMETHOD

	NO.	ORIGINAL METHOD % DILUTION OF $CaCl_2$	MICROMETHOD 0.5% $CaCl_2$	
Widening of K.B. Proliferative (Fibrous) Processes	10	0.01	2 drops	Shift to right
	9	0.02	4 drops	
	8	0.03	6 drops	
	7	0.04	8 drops	
Normal K.B.	6	0.05	10 drops	Normal Zone
	5	0.06	12 drops	
Narrowing of K.B. Exudative Processes	4	0.07	14 drops	Shift to left
	3	0.08	16 drops	
	2	0.08	18 drops	
	1	0.1	20 drops	

In cases of fibrous or proliferative processes, as cirrhosis of the liver, fibrous type of lung tuberculosis, and chronic pneumonia, there is a widening of the K.B. (shift to the right) i.e., flocculation may be present at the eighth tube or higher.

Dees[11] found that this reaction is of assistance in the differential diagnosis between uncomplicated and gangrenous appendicitis. In the uncomplicated case, the K.B. is slightly less than normal, but in the gangrenous condition, the narrowing is marked.

From our experience it is our opinion that the importance of the Weltmann reaction is in the tracing of the course and complications of the disease. For this purpose frequent repetitions of this test during the course of the disease must be carried out, and these require frequent venipunctures.

Havas[12] tried to introduce a micromethod, but his technique was too complicated.

This publication presents a micromethod for the simplification of the Weltmann reaction.

TECHNIQUE

The necessary equipment consists of:

(1) Microcentrifuge tubes of 1 c.c. capacity.
(2) Special micropipettes, i.e., a blood collecting pipette as used for the micromethod.

(3) A 0.1 c.c. graduated pipette. The necessary reagent is a 0.5 per cent calcium chloride solution.

Five-tenths cubic centimeter of blood, i.e., 10 drops, is drawn with the blood pipette, placed in the microcentrifuge tube, and centrifuged for fifteen minutes. One-tenth cubic centimeter of the nonhemolyzed serum is pipetted from this tube into a clean Wassermann tube. To it are added 4.9 c.c. of distilled water and 2 drops of the 0.5 per cent calcium chloride solution.

There is now the same concentration as will be found in the tenth tube in the original method. (See table for comparison of the concentrations of the original method and the micromethod.) The contents of the tube are well mixed and boiled over an open flame for one to one and one-half minutes. During this time the tube is continually shaken and observed for flocculation.

If several sera are to be examined, several tubes with the various sera and the necessary solution can be prepared. These tubes are then placed in a boiling water bath for five minutes and observed for flocculation, using either method. If no coagulation is present, 2 more drops of 0.5 per cent calcium chloride are added and the contents are boiled.

In a normal case flocculation occurs after the addition of 8 to 10 drops of the 0.5 per cent solution of calcium chloride, which compares to the sixth or seventh tube in the original method, that is, a concentration of 0.04 to 0.05 per cent.

In comparing the results of the two methods, we found that they check throughout.

The advantages of the micromethod are: (1) less blood is needed, and thus the necessary amount of serum can be obtained by just a puncture of the finger or ear; (2) venipuncture is not necessary, and for this reason daily repetition of the test is possible without much discomfort to the patient; (3) less time is expended in carrying out this method.

SUMMARY

A micromethod for the Weltmann reaction is reported. The importance of the test for the diagnosis, course, and prognosis of the different diseases are here stressed. The advantages of this micromethod are mentioned.

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CONCERNING THE CHOICE OF COMPLEMENT-ANTIGEN COMBINATION FOR USE IN THE KOLMER COMPLEMENT FIXATION TEST*

I. A Pretesting Method for Complement Selection

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ALMOST as important as the method of preparing antigen is the selection of satisfactory guinea pig serum to be used as complement in the Kolmer complement fixation test for syphilis. Apparently the optimal quality of complement may vary with each individual guinea pig and also may vary from time to time. A complement that is satisfactory with one lot of Kolmer antigen may give unsatisfactory results when used with another Kolmer antigen. Certain complement-antigen combinations which are unsatisfactory may be detected and eliminated by a preliminary control method for testing the combination of reagents.

As reported by Giordano and Carlson,[1] and Kolmer,[2] some guinea pig sera possess a component that is capable of fixing complement in the presence of Kolmer antigen at 6° to 10° C. That some complement sera may also give atypical, zonal false positive reactions with negative human serum has also been reported.[2]

Since this nonspecific reaction of antigen and complement may not occur at 37° C. (during the complement titration), a clue to the presence of this undesirable characteristic of some guinea pig sera is not always available until after the overnight incubation period of the test. The antigen control tube can be expected to show partial or complete inhibition of hemolysis after one hour of secondary incubation at 37° C., when complement that is readily deviated by antigen alone is employed. Under such test conditions the spinal fluid tests may be falsely positive, and some quantitative serum tests may be negative in 0.2 c.c. amounts with positive reactions in the tubes containing smaller amounts of serum. The elimination, from a herd, of guinea pigs whose sera give this type of reaction has not always solved this problem, since previously satisfactory guinea pigs have been found to yield serum having this undesirable property

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at a later date. Nor does the pooling of sera from many guinea pigs yield a complement of usable reactivity in all instances.

The results of the following studies have guided the formulation of a pretesting procedure which will enable a laboratory, using either lyophilized or fresh complement, to select a satisfactory complement for use with the antigen employed in its Kolmer test.

TECHNIQUE

The Kolmer complement fixation test procedure and the test reagents used are those described in Venereal Disease Information Supplement No. 11, 1940. The guinea pigs used were healthy, male animals on a mixed diet of oats, hay, cabbage, and carrots.

PROCEDURE

The blood samples were obtained by cardiac puncture from each of 20 guinea pigs, the second and third blood samples were obtained fourteen and twenty-nine days after the initial bleeding. Sera from these bloods were tested with and without antigen to determine the degree of variability in nonspecific complement destruction that might be encountered. Two four-tube sets, containing 1.0 c.c. dilutions of guinea pig serum at 1:30, 1:33, 1:37, and 1:43, were used for each animal tested. To one of these sets of tubes was added a test dose of Kolmer antigen dilution (0.5 c.c.) plus 0.5 c.c. of saline, and to the other set, 1.0 c.c. of saline containing no antigen. All tubes were then placed in the refrigerator at 6° C. for sixteen hours. After a further incubation of ten minutes at 37° C., two units of hemolysin and 0.5 c.c. of cell suspension were added to each tube, and readings were made after a secondary incubation of one hour at 37° C. The results of these testings are listed in Table I.

In Table I the tubes containing antigen are equivalent to the antigen control tubes that would accompany a test at that complement titer, and it is noted that the destruction of complement occurring in these tubes varied within broad limits. Samples of serum, obtained from some guinea pigs on different dates, showed varying degrees of susceptibility to nonspecific destruction of complement. These results point to the fact that some guinea pigs may yield a satisfactory complement at one time and not at another. However, when the three test results are observed, it is evident that all the animals in this group did not show the same degree of serum change nor were all the changes in the same time direction. For this reason, it has been found necessary to test each complement serum at the time of bleeding rather than to rely on any previous test finding.

In order to ascertain the role of antigen in this nonspecific destruction of complement at 6° C., serial dilutions of complement were tested for hemolytic activity after having been in contact with test doses of each of two lots of Kolmer antigen (X and Y) at this temperature for sixteen hours. Control tubes, containing no antigen, were included in this test, as presented in Table II.

The dilutions of complement (1:37, 1:50, and 1:75) used in testing the anticomplementary activities of these two antigens allowed a marked differentiation to be evidenced between them. Since each of these antigens was made under similar conditions, but from different lots of beef heart powder, and since both

TABLE I

RESULTS OF ANTICOMPLEMENTARY EFFECT OF KOLMER ANTIGEN ON THE COMPLEMENT SERA OF 20 GUINEA PIGS BLED AT THREE INTERVALS

GUINEA PIG	COMPLEMENT DILUTIONS BLED AND TESTED											
	AUGUST 6				AUGUST 20				SEPTEMBER 4			
	1:30	1:33	1:37	1:43	1:30	1:33	1:37	1:43	1:30	1:33	1:37	1:43
1	3	3	4	4	4	4	4	4	–	–	–	±
2	4	4	4	4	4	4	4	4	4	4	4	4
3	–	–	–	±	–	–	–	±	–	–	–	±
4	–	–	–	–	–	–	–	–	–	–	–	–
5	2	3	4	4	3	4	4	4	–	–	±	3
6	±	1	3	4	–	–	±	±	–	–	2	3
7	4	4	4	4	1	2	3	4	–	–	1	3
8	±	1	2	3	–	–	–	–	–	–	–	–
9	4	4	4	4	4	4	4	4	4	4	4	4
10	±	1	2	3	–	–	–	–	–	–	–	–
11	±	±	±	±	–	–	–	–	–	–	–	–
12	–	–	–	–	±	1	1	2	±	±	4	4
13	–	–	–	–	–	–	–	–	–	–	–	–
14	3	3	3	3	1	±	±	±	–	–	–	–
15	–	–	–	±	–	–	–	–	–	–	–	–
16	–	–	–	–	–	–	–	–	–	–	–	–
17	±	±	1	3	–	–	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	–	–	–
19	–	–	–	–	–	–	–	–	–	–	–	–
20	–	–	–	–	–	–	–	–	–	–	–	–

Each of the above readings was made on the tubes containing a test dose of Kolmer antigen (0.5 c.c. of 1:640 dilution). Duplicate control tubes of each of the complement dilutions, containing no antigen, gave complete hemolysis in every instance.

These tests were performed with a primary incubation period of sixteen hours at 8° C., plus 10 minutes at 37° C., and readings were made after a secondary incubation period of one hour at 37° C.

The same lot of Kolmer antigen was used throughout these three testings.

TABLE II

RESULTS OF TESTS WITH THE COMPLEMENT SERA OF 30 GUINEA PIGS SHOWING THE ANTICOMPLEMENTARY EFFECT OF TWO LOTS OF KOLMER ANTIGEN

GUINEA PIG	COMPLEMENT DILUTION (1.0 C.C.)								
	KOLMER ANTIGEN (Y)*			KOLMER ANTIGEN (X)*			NO ANTIGEN ADDED		
	1:37	1:50	1:75	1:37	1:50	1:75	1:37	1:50	1:75
1	–	2	4	–	–	–	–	–	–
2	1	3	4	–	–	–	–	–	–
3	–	±	3	–	–	–	–	–	–
4	3	4	4	–	–	±	–	–	–
5	±	4	4	–	–	–	–	–	–
6	±	1	3	–	–	–	–	–	–
7	–	1	4	–	–	–	–	–	–
8	–	–	1	–	–	–	–	–	–
9	–	±	1	–	–	–	–	–	–
10	±	3	4	–	–	–	–	–	–
11	–	1	4	–	–	–	–	–	–
12	–	4	4	–	–	–	–	–	–
13	4	4	4	–	–	–	–	–	–
14 to 30 incl.	–	–	–	–	–	–	–	–	–

*Each of these antigens was used in the test dose (0.5 c.c. of 1:640 dilution) amounts.

These tests were performed with a primary incubation period of sixteen hours at 8° C. plus ten minutes at 37° C. Cell suspension (0.5 c.c. of 2 per cent suspension) and 0.5 c.c. of hemolysin, containing 2 units, was then added to all tubes, and final readings were made after a secondary incubation period of one hour at 37° C.

TABLE III

RESULTS OF KOLMER COMPLEMENT FIXATION TESTS PERFORMED ON 20 HUMAN SERA AND 10 SPINAL FLUIDS EMPLOYING TWO LOTS OF KOLMER ANTIGEN AND TWO LOTS OF LYOPHILIZED COMPLEMENT SERA

NUMBER	KOLMER ANTIGEN X PLUS COMPLEMENT B	KOLMER ANTIGEN Y PLUS COMPLEMENT B	KOLMER ANTIGEN X PLUS COMPLEMENT A	KOLMER ANTIGEN Y PLUS COMPLEMENT A
		Sera		
1	3 2 ± – –	2 2 1 ± –	4 3 2 ± –	1 2 3 4 4
2	– – – – –	– – – – –	– – – – –	– – – ± 3
3	– – – – –	– – – – –	– – – – –	– – ± 1 3
4	– – – – –	– – – – –	– – – – –	– – ± 2 4
5	– – – – –	– – – – –	– – – – –	– – – – –
6	– – – – –	– – – – –	– – – – –	– – – ± 4
7	– – – – –	– – – – –	– – – – –	– – – ± 4
8	– – – – –	– – – – –	– – – – –	– – – ± 4
9	4 ± – – –	3 ± – – –	4 2 ± – –	± ± ± 1 4
10	4 4 4 4 4	4 4 4 4 4	4 4 4 4 4	4 4 4 4 4
11	– – – – –	– – – – –	– – – – –	– – – ± 4
12	4 4 ± – –	4 3 ± – –	4 4 2 – –	3 2 1 1 2
13	– – – – –	– – – – –	– – – – –	– – – ± 1
14	4 2 – – –	4 3 ± – –	4 3 ± – –	4 2 1 1 2
15	– – – – –	– – – – –	– – – – –	– – – – 1
16	– – – – –	– – – – –	– – – – –	– – – – –
17	± ± – – –	± ± – – –	± ± ± – –	– – – ± 1
18	– – – – –	– – – – –	– – – – –	– – – – 1
19	4 4 4 4 4	4 4 4 4 4	4 4 4 4 4	4 4 4 4 4
20	– – – – –	– – – – –	– – – – –	– – – – –
		Spinal Fluids		
1	± – – – –	± ± – – –	± ± ± – –	1 2 3 4 4
2	4 2 – – –	4 4 – – –	4 4 2 – –	4 4 4 4 4
3	± – – – –	± – – – –	3 ± – – –	4 4 4 4 4
4	4 4 – – –	4 4 3 1 –	4 4 4 3 2	4 4 4 4 4
5	± – – – –	1 ± – – –	2 ± – – –	4 4 4 4 4
6	– – – – –	– – – – –	– – – – –	4 4 4 4 4
7	– – – – –	– – – – –	– – – – –	4 4 4 4 4
8	– – – – –	– – – – –	– – – – –	4 4 4 4 4
9	– – – – –	– – – – –	– – – – –	4 4 4 4 4
10	– – – – –	– – – – –	– – – – –	4 4 4 4 4
Complement titer	1:33	1:33	1:33	1:33
Hemolysin titer	1:3,000	1:3,000	1:3,000	1:3,000
Hemolytic control	Clear 10 min.	Clear 10 min.	Clear 10 min.	Clear 10 min.
Antigen control	Clear 15 min.	Clear 15 min.	Clear 23 min.	4+ after 60 min.

The readings were made ten minutes after the antigen controls cleared in each of the first three groups above. Where Kolmer antigen Y and complement B were used, the tests were read after one hour of secondary incubation.

TABLE IV

A PRETEST METHOD FOR THE SELECTION OF SUITABLE COMPLEMENT FOR THE KOLMER COMPLEMENT FIXATION TEST

TUBES	1.0 C.C. OF COMPLEMENT DILUTED 1:30	TEST DOSE OF KOLMER ANTIGEN	KOLMER SALINE	Refrigerator at 6° to 8° C. for 16 to 18 hours plus 10 min. in 37° C. water bath	2 UNITS OF HEMOLYSIN	2% CELL SUSPENSION	Readings made after one hour in 37° C. water bath
1	0.8	0.5	0.7		0.5	0.5	
2	0.6	0.5	0.9		0.5	0.5	
3	0.4	0.5	1.1		0.5	0.5	
4	0.8	None	1.2		0.5	0.5	
5	0.6	None	1.4		0.5	0.5	
6	0.4	None	1.6		0.5	0.5	

Mix 0.15 c.c. of guinea pig serum with 4.35 c.c. of Kolmer saline for 1:30 complement dilution.

antigens met with the other test demands and gave maximum fixability at the same dilution (1:640), a comparative evaluation under actual test conditions was arranged. For this purpose, serum from guinea pigs No. 1 to 13 (Table II), inclusive, was pooled and lyophilized, and will be referred to as complement A, and serum from guinea pigs No. 14 to 30 (Table II), inclusive, was pooled and lyophilized, and will be designated as complement B.

A series of 20 blood samples (7 positive and 13 negative) and 10 spinal fluids (5 positive and 5 negative) were tested simultaneously in four separate groups. Each of the antigens (X and Y) was used with each of the complements (A and B), giving the results recorded in Table III.

The tests in which antigen X and complement B were used, as well as those containing antigen Y plus complement B, and antigen X plus complement A, reacted in an orderly manner, i.e., the controls (hemolytic system and antigen) cleared in less than thirty minutes, and the tests were readable in less than one hour of secondary incubation. Antigen Y and complement A proved to be an unsatisfactory combination, since the antigen control tube did not clear, the spinal fluids gave false positive reactions, and many of the serum specimens gave atypical, zonal reactions.

These observations have aided in the formulation of the following preliminary pretest control method for observing the behavior of the complement-antigen combinations. Each guinea pig serum used, whether as fresh complement or as part of a lyophilized lot, is pretested with the antigen with which it will later be used in the Kolmer test. This may be accomplished by bleeding guinea pigs into individual test tubes, allowing these tubes to remain at room temperature until a firm clot is formed, and then placing them in the refrigerator for a few hours. After this period the clots are loosened with a wooden applicator, centrifuged at high speed for a few minutes, and the 0.15 c.c. serum needed for pretesting is removed from each tube. The tubes containing clot and serum are then placed in the refrigerator until the following morning. This testing method is outlined in Table IV.

Guinea pig sera that gave complete hemolysis in all six tubes of this test have been found to be of good quality and to have the degree of hemolytic activity required by the test author (1:30 to 1:43 for two full units). A guinea pig serum showing an equal inhibition of hemolysis in both the antigen tubes, and those containing no antigen, may be considered usable but will usually be found to have a lower hemolytic activity, as determined by the complement titration on the following day. Any sera causing a greater inhibition of hemolysis in the presence of antigen than is found in the tubes containing no antigen, may be considered as unsatisfactory when used with the testing antigen.

Using this pretesting method, more than twenty lots of lyophilized complement, satisfactory for use in the Kolmer complement fixation test, have been prepared. Each of these lots of complement was pooled from 25 to 31 guinea pigs, obtained from random herds from several breeding sources. Fifty lots of fresh complement (5 guinea pigs each), chosen in the same manner, have all been found to be of satisfactory reactivity.

DISCUSSION

Kolmer has stated that ". . . prezone and nonspecific complement-fixation tests are due to factors involving the complement employed . . . ," and it appears that the degree of this reactivity may also be influenced by some antigen component. Whereas antigen Y with complement A proved to be an unsatisfactory combination, antigen Y with complement B, and complement A with antigen X, gave satisfactory test results. Therefore, antigen Y or complement A can be considered usable if combined with a suitable complement or antigen, respectively, but are entirely unsatisfactory when used together.

When fresh complement is employed, the preliminary antigen-complement combinations of the pretest procedure can be placed in the refrigerator the afternoon the blood is drawn. This can be arranged by removing the small amount of serum needed for testing from each tube and allowing the remainder to stay on the clot until the following morning. After a reading has been made on each serum, sera found to have satisfactory reactivity can be pooled, titrated, and used without further delay. When large quantities of serum are to be lyophilized, a small amount of blood (0.5 c.c. is adequate) may be obtained from each guinea pig and tested the day before exsanguination. However, the routine used for fresh complement can be applied to complement for lyophilization if the whole blood is kept at a low temperature, during the overnight storage period, and the dehydration process is started as soon as possible after the pooling of the satisfactory sera. This pretesting procedure for complement selection is not offered as a substitute for any part of the Kolmer titrations, but rather as an adjunct to the complement titration.

SUMMARY

1. The suitability of a guinea pig complement for use with a specific Kolmer antigen may vary, within broad limits, in some guinea pigs during fourteen- to twenty-three-day intervals.

2. As demonstrated by a sixteen-hour incubation period at 6° C., the anticomplementary action of two lots of Kolmer antigen, having identical test dose titers, was not similar on all complements tested.

3. A pretest method for complement selection is described.

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CHEMICAL

THE CHOLINE CONTENT OF BIOLOGICAL FLUIDS*

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IN THE course of some studies on the presence and significance of choline in cortico-adrenal extracts[1] a survey was made of the available methods for the quantitative determination of choline in relatively small amounts of fluid. Although the widespread occurrence of choline and its compounds in the normal organism had been regarded as some indication of functional significance,[1] and although choline and its compounds have become increasingly important in recent years because of the unique role of an essential dietary factor as well as a hormone, little or no quantitative data can be found on the choline content of the various biological fluids.

The biological methods for choline determination depend on the conversion of the choline to its much more powerful acetic ester, and the subsequent assay of the latter on some test object by comparison with standard acetylcholine solutions. Chemically, choline forms precipitates with many reagents, but of these only three (the double salts with iodine, platinic chloride, and mercuric chloride) can be formed when the choline is in dilution of 1:2,000,000.

The original method of Sharpe[2] was based on the work of Stanek,[3] who precipitated choline as the unstable periodide and estimated the iodine by titration with sodium thiosulfate. Roman,[4] however, suggested the use of a stronger solution of iodine, precipitating the choline as the enneaiodide ($C_5H_{14}NOI.I_8$). Many investigators have used some modification of the latter procedure. The objections of this procedure are: (a) It requires at least 10 to 20 c.c. of blood. (b) Extraction by heating on a boiling water bath (presumably to split choline esters) involves the loss of free choline by decomposition, and increases the possibility of lecithin hydrolysis, resulting in newly liberated choline. (c) Dialysis for twenty-four hours against distilled water and reduction in volume of the dialysate offer excellent possibilities for the loss of choline, even up to 100 per cent. (d) Choline periodide and enneaiodide are unstable products and losses occur during evaporation to small volume. (e) The ice-cold water (0 to 1° C.) used to wash away the excess iodine dissolves some of the soluble precipitate. (f) The reagent likewise precipitates ammonium salts, stachydrine, and other extractives which cannot be removed completely without likewise affecting the precipitate.

Precipitation of the choline as the double salt with platinic chloride or Reinecke's acid may be used, and the resulting precipitates may be purified and weighed as such, checked by melting point determinations, analyzed for nitrogen, or converted to some other salt (e.g., gold salt) which can be assayed

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biologically. In any case, large amounts of the original choline-containing fluid would be required for such procedure.

Addition of a saturated solution of mercuric chloride in absolute alcohol to an alcoholic solution of choline results in the formation of a stable precipitate of choline mercury chloride ($C_5H_{14}ONCl.\ 6HgCl_2$), melting point 242°-243°. The double salt separates out as extremely insoluble needles which can be centrifuged and thoroughly washed without loss. The mercury content can then be determined by volumetric analysis.

METHOD

The method used in the present study is essentially that of Ambo and Aoki[5] and, with modifications, is given in detail as follows: Two to 5 c.c. of blood are measured into 10 to 25 c.c. of 95 per cent alcohol containing 2 to 3 drops of acid alcohol, shaken, and allowed to stand for twenty-four hours for precipitation and extraction. The mixture is then filtered, the residue is washed well with a fine stream of 95 per cent alcohol, and the total filtrate is carefully evaporated under reduced pressure to almost dryness. It is important to avoid splashing and to keep the temperature of the bath below 40° C. at all times. The residue (a few drops) is extracted three times with petroleum ether to remove fat and phosphatides, the petroleum ether being removed in each case by centrifuging and drawing off the supernatant fluid with a medicine dropper drawn out to a fine capillary. The remaining small amount of liquid is then evaporated to dryness under reduced pressure, the residue is extracted three times with small amounts of cold absolute alcohol and filtered through a very small funnel into a 50 c.c. flask (thus removing the material insoluble in cold alcohol). The filtrate is then brought to a very small volume (0.1 to 0.2 c.c.) under reduced pressure, and 1 to 2 c.c. of a saturated solution of mercuric chloride in absolute alcohol is then added. The flask is shaken well and placed in the refrigerator for twenty-four hours for complete precipitation of the choline mercury chloride.

The contents of the flasks (the precipitate and the excess mercuric chloride in absolute alcohol) are then transferred to centrifuge tubes and centrifuged rapidly at 2,800 r.p.m., and the clear, supernatant fluid is removed with a capillary medicine dropper and discarded. Absolute ether is used to wash and transfer the contents of the flask to the centrifuge tube. The precipitate is then washed six times with absolute ether, with centrifuging and removal of the supernatant fluid after each washing. It is essential that the precipitate and its container be washed completely free from excess mercuric chloride. The precipitate is then dissolved in 1 c.c. of 10 per cent potassium iodide and transferred to a flask, to which are then added 1 c.c. of 15 per cent potassium hydroxide, 3 drops of mucilage of acacia (U.S.P.) and, while shaking, 1 c.c. of 11.5 per cent formaldehyde. The free mercury separates out in fine suspension, which is first shaken well and then allowed to stand for five minutes. The mixture is then acidified with 6 c.c. of 20 per cent glacial acetic acid and 2 c.c. of 0.005 N potassium iodate solution are added from a microburette. After shaking gently to enable solution of mercury (the freed iodine combined with mercury), the excess potassium iodate is titrated with 0.005 N sodium thiosulfate, with

starch as an indicator. The 0.005 normal solutions of potassium iodate and sodium thiosulfate are freshly prepared from standardized 0.1 N stock solutions; the former are standardized at the time of titration of the regularly prepared samples.

Calculation: Each 0.1 c.c. of 0.005 N potassium iodate used combines with 0.067 mg. mercuric chloride, which is equivalent to 0.0058 mg. choline chloride containing 0.005 mg. choline.

There are several advantages of this method: (1) A small amount of the original fluid is used, so that two or more analyses may be performed on the same sample. (2) The disadvantages of choline instability in aqueous solution, and its volatility on reduction to small volume on a water bath, are avoided by the use of absolute alcohol as a solvent and distillation under reduced pressure for concentration to small volume. (3) The free choline is separated from interfering material without loss and without the possibility of liberation of bound choline from lecithin, sphingomyelin, etc. (4) The precipitant is effective even in a 1:2,000,000 dilution of choline and forms a stable, insoluble precipitate. (5) The mercury content of the precipitate may be accurately determined by standard quantitative inorganic procedure, from which the choline content is readily calculated.

RESULTS

The recovery of choline from standard solutions of choline chloride in 95 per cent alcohol is shown in Table I. The range of recovery is from 87.6 to 118.6 per cent; the average recovery for the series is 99.5 per cent.

TABLE I

RECOVERY OF CHOLINE FROM STANDARD ALCOHOLIC SOLUTIONS

CHOLINE PRESENT (GAMMA)	CHOLINE RECOVERED	
	(GAMMA)	(PER CENT)
14.3	13.5	94.4
29.0	27.0	93.2
29.0	26.0	89.7
35.9	37.5	104.3
33.0	37.0	112.0
33.0	35.0	106.0
86.0	76.0	88.4
104.0	102.0	98.1
113.0	99.0	87.6
120.0	106.0	88.4
125.0	120.0	96.0
136.0	140.0	102.8
274.0	245.0	89.4
431.0	407.0	94.5
431.0	467.0	108.3
666.0	790.0	118.6
666.0	754.0	113.2
666.0	701.0	105.2
856.0	842.0	98.3
862.0	870.0	100.9
862.0	864.0	100.2

Two standard choline chloride solutions (containing 6.66 and 19.83 mg. of choline per cubic centimeter) were submitted for nitrogen analysis in the chemistry laboratory of Billings Hospital. On the basis of the reported nitrogen content, these solutions had choline equivalents of 6.52 and 19.45 mg. per cubic

centimeter, 97.9 and 98.1 per cent, respectively, of the calculated choline content. These solutions were subsequently used to determine the yield of choline mercury chloride from solutions of known choline content (average yield 98.4 per cent), and the per cent of choline recovery from known amounts of choline mercury chloride dissolved in 10 per cent potassium iodide solution. In a series of 11 control experiments made on four such potassium iodide solutions (whose choline equivalents were 1.208, 0.683, 0.418, and 0.280 mg. per cubic centimeter) the range of choline recovery was from 88.3 to 102.3 per cent; the average recovery for the series was 93.4 per cent.

TABLE II

RECOVERY OF CHOLINE ADDED TO BIOLOGICAL FLUIDS

ORIGINAL FLUID	CHOLINE CONTENT (GAMMA)	CHOLINE ADDED (GAMMA)	TOTAL CHOLINE PRESENT (GAMMA)	TOTAL CHOLINE RECOVERED	
				(GAMMA)	(PER CENT)
1 c.c. serum ♂	11.6	29.6*	41.2	37.8	91.7
2 c.c. blood ♂	25.6	43.0	68.6	61.2	89.1
2 c.c. blood ♂	37.4	43.0	80.4	70.7	87.9
2 c.c. blood ♀	28.4	86.0	114.4	101.8	89.0
2 c.c. blood ♀	30.1	86.0	116.1	106.0	91.3
3 c.c. blood ♀	37.8	86.0	123.8	112.2	90.6
1 c.c. menstrual blood	32.2	215.0	247.2	211.3	85.5
2 c.c. cow's milk	162.0	323.8	485.8	425.9	87.7
1 c.c. human semen	337.0	323.8	660.8	604.6	91.5
1 c.c. corticoadrenal extract	1324.0	772.5	2096.5	2120.0	101.1

*1 c.c. of a 1:10 dilution of human semen.

Table II gives the results obtained in control experiments in which known amounts of standard alcoholic choline chloride solutions were added to various biological fluids. The range of total choline recovery was from 85.5 to 101.1 per cent; the average recovery for the series was 90.5 per cent.

TABLE III

CHOLINE CONTENT OF BIOLOGICAL FLUIDS

NO.	MATERIAL	NO. SAMPLES	HIGHEST VALUE (MG./LITER)	LOWEST VALUE (MG./LITER)	AVERAGE VALUE (MG./LITER)
1	Human saliva ♂ (a)	12	14.4	8.6	11.6
2	Human saliva ♂ (b)	7	9.9	4.7	6.5
3	1 and 2 combined	19	14.4	4.7	9.7
4	Human saliva ♀ (a)	75	36.4	6.2	16.9
5	Human blood ♂ (c)	8	17.1	10.3	13.2
6	Human blood ♂ (d)	10	22.8	8.5	13.5
7	5 and 6 combined	18	22.8	8.5	13.4
8	Human blood ♀ (d)	19	21.8	9.4	14.6
9	Human blood, pregnant ♀	62	19.9	9.9	13.1
10	Human semen	17	477.0	234.0	341.0
11	Dog bladder bile	7	380.0	78.0	250.0
12	Dog lymph	3	23.5	14.2	18.2

(a) Patients chewed paraffin and collected samples in bottles.
(b) Dilute saliva collected by continuous use of dental siphon.
(c) Normal persons.
(d) Patients from Out-Patient Department of Billings Hospital.

The results of choline analyses on most of the biological fluids studied are summarized in Table III. All analyses were done in duplicate; in many cases they were repeatedly performed on the same sample to determine the degree of

accuracy of triplicate and quadruplicate analyses on the same sample through a wide range of choline concentrations. The total number of analyses, including those on standard solutions and miscellaneous samples, exceeded 650. Detailed studies on blood choline variations in pregnancy and salivary choline variations during the menstrual cycle will be presented in subsequent papers.

SUMMARY

1. A modified chemical method for the quantitative determination of choline in small amounts of biological fluids has been described in detail.

2. Control studies have been made to show choline recovery from standard alcoholic choline chloride solutions, from solutions of known choline mercury chloride content, and from biological fluids to which known amounts of choline were added.

3. The choline content of 220 samples of various biological fluids is reported.

Grateful acknowledgment is made to Dr. A. J. Carlson for his cooperation throughout the course of this work.

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EFFECT OF ADDED SALTS ON RECOVERY OF HIPPURIC ACID FROM URINE*

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IT HAS generally been found that hippuric acid varies in solubility in different urines, the loss on recovery being too variable for reasonably accurate clinical results of the Quick liver function test as originally outlined.[1, 2] Efforts have been made to reduce this loss, at least to a constant value. Several years ago Berg and Mattill[3] recommended the use of ammonium sulfate in student laboratory procedure for the isolation of hippuric acid from urine. A modification of their procedure has been used in this laboratory in liver function tests for three years with excellent results. Quick revised his analytical method to include the use of ammonium sulfate,[4] but has not published the experimental work.[5] Sodium chloride[6] has also been employed to reduce the hippuric acid solubility in urine, but the results are not directly applicable to the conditions of the liver function test where recovery losses need to be known for a urine solution of a salt of the hippuric acid.

This paper presents a study of the effect of the addition of ammonium sulfate and some other salts on the loss in recovery of hippuric acid from urine in gravimetric procedure. Volumetric analysis is less desirable, since added salts affect the titration values, and it has been found far less precise in the hands of a number of technicians.

EXPERIMENTAL

Fresh, clear urine specimens were picked at random from routine hospital specimens in order to have representative results. Those containing more than a trace of albumin, or having unusual color, were rejected. To simulate the chemical composition of urine as it is passed in Quick liver function tests, hippuric acid (0.80 Gm.) was added as a solution of the sodium salt, prepared by neutralizing the acid with dilute sodium hydroxide. The desired amount of a salt to be tested for its effect in reducing the solubility of hippuric acid was then dissolved in the urine, and the mixture was run through a paper of rapid filtering quality, or centrifuged to remove the solids which separated. A solid phase which contained no hippurate separated from all urines when sufficient amounts of a salt were added. Therefore, the minimum quantity of a salt to be used was the amount necessary to condition hippurate-free urine to the point where no further separation of solids occurred upon acidification.

After filtration 1 c.c. of concentrated sulfuric acid was added for each 100 c.c. of urine used. Added sodium hippurate solution was considered part of the urine volume. The solution was stirred until several minutes after hippuric acid precipitated. In order to achieve constant results it was necessary to start

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crystallization by friction between the beaker and stirring rod. One to four hours later the precipitate was collected quantitatively on a weighed filter paper over suction. The filtrate was used to wash the beaker. The precipitate and the inside of the funnel were washed with 15 c.c. of distilled water (25° C.) from a medicine dropper. This left less than 0.01 Gm. of the precipitating agent on the filter and had a negligible effect on the quantity of hippuric acid. Weight of the precipitate was taken after it had dried twelve hours at 37° C. A 5.5 cm. No. 4 Whatman filter on a 67 mm. Buchner porcelain funnel fitted into a side-neck test tube was found convenient, because all the solid settled on the paper, and any loss could be seen in the tube and recovered.

DISCUSSION AND RESULTS

By this method the true solubility of hippuric acid in treated urine was not determined, but the loss incurred in actual procedure was ascertained. The variations in recovery from different specimens is probably largely due to the inability of added salts to abate entirely the effects of urinary colloids on crystallization.

TABLE I

LOSS OF HIPPURIC ACID IN URINES TO WHICH SALTS HAVE BEEN ADDED TO FACILITATE PRECIPITATION

SPECIMEN	URINE VOLUME (C.C)	HIPPURIC ACID LOSS (GM./100 C.C.)	SPECIMEN	URINE VOLUME (C.C)	HIPPURIC ACID LOSS (GM./100 C.C.)
Ammonium Sulfate			*Magnesium Sulfate*		
1	75	0.116	22	75	0.140
2	75	0.120	23	75	0.180
3	75	0.080	24	75	0.152
4	75	0.124	25	75	0.168
5	75	0.146	26	75	0.140
6	75	0.140	27	75	0.121
7	75	0.125	28	75	0.149
8	75	0.066	29	75	0.152
9	75	0.105	30	100	0.052
10	75	0.140	31	100	0.076
11	75	0.144	32	200	0.128
12	100	0.085	33	200	0.139
13	100	0.067	34	200	0.056
14	100	0.067	35	200	0.121
15	150	0.078	Average		0.127
16	200	0.091			
17	200	0.090	*Sodium Sulfate*		
18	200	0.036	36	75	0.170
19	200	0.105	37	75	0.166
20	200	0.056	38	75	0.157
21	200	0.113	39	75	0.139
Average		0.100	Average		0.158

Table I gives the hippuric acid loss for 21 different urine specimens to which 35 Gm. of ammonium sulfate were added for each 100 c.c. volume. This is the minimum quantity of that salt practical for use; more of it has no advantage. For clinical accuracy 0.1 Gm. can be added to the amount of hippuric acid recovered from each 100 c.c. of urine by the method outlined here. This holds true for urine volumes to 200 c.c. without concentration. However, the necessity of concentrating urine from patients with copious output is not obviated.

Magnesium sulfate is a more economical salt to add to urine. Experimental results with the use of this salt show slightly greater average loss per 100 c.c.; nevertheless, the data in Table I indicate it is a good agent to use. Fifty grams per 100 c.c. of urine are necessary. Magnesium sulfate in this concentration is still far enough from the saturation point that it does not readily crystallize out. An average loss of 0.127 Gm. per 100 c.c. of urine is to be added to the hippuric acid recovery if magnesium sulfate is employed at 50 Gm. per 100 c.c.

Four determinations with 35 Gm. of sodium sulfate per 100 c.c. of urine gave an average loss of 0.158 Gm. of hippuric acid per 100 c.c. Addition of more of this salt was found impractical.

Thirty grams of sodium chloride per 100 c.c. of urine reduced the loss to 0.1 Gm. or less. Since it is more difficult than the other salts used to wash from the hippuric acid, it probably contributed to the recovery in the trials made. Moreover, the effective concentration of salt is so close to its saturation point in urine that difficulty is experienced with its crystallizing from solution and on the filter. Its use in either volumetric or gravimetric method for hippuric acid lengthens the procedure because heat is necessary to effect solution, and this must be followed by a cooling period.

SUMMARY

Of four salts tested for reducing the solubility of hippuric acid in urine to a constant value, ammonium sulfate was found to be the best. The loss was reduced to an average of 0.1 Gm. per 100 c.c. of urine under the conditions of the Quick liver function test, using gravimetric procedure.

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A NOTE ON THE USE OF TÖPFER'S REAGENT IN GASTRIC ANALYSIS AFTER TEST MEALS OF MILK, CREAM, OR SOYBEAN BISCUITS*

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IN TESTING a patient with duodenal ulcer with various foods to see whether any of them would decrease the gastric acidity, it was found that milk would give about one-half as much free acid as the bread test meal, using Töpfer's reagent as the indicator. Soybean biscuits also decreased the acidity. Casein and whey were then used, but neither of these showed any apparent change in the total free acid. Finally, sweet cream was fed after which no free acid was indicated by Töpfer's in the fractional test meal. Likewise, Töpfer's failed to indicate change of acidity when an excess of hydrochloric acid was added to cream in vitro.[1] Since the salts of milk should give it more buffering action than cream, the problem was investigated further.

Preliminary experiments on the titration of milk and cream with hydrochloric acid, using Töpfer's reagent as the indicator, suggested that the indicator did not function properly in the presence of these substances. To check the action of the indicator, potentiometric titrations were carried out using a Leeds and Northrup pH meter with glass electrode. Stepwise additions of N/10 hydrochloric acid and sodium hydroxide produced the expected changes in pH. With phenolphthalein as the indicator sodium hydroxide caused the solution to turn pink at pH 8.5. However, with Töpfer's reagent as the indicator the solution remained yellow even after the acidity was lowered to pH 1.5. Further addition of 2 ml. of concentrated sulfuric acid caused the solution to turn red, which should have occurred at pH 2.9.

A sample of milk was acidified with N/10 hydrochloric acid to pH 2.5. Töpfer's reagent was added, and the indicator was still yellow. After centrifuging it was noted that the fat and protein layer had a yellow color, while the milk serum was slightly pink. On adding one more drop of Töpfer's reagent to the milk serum it turned red.

These results indicate that the fat and/or proteins of the milk interfere with the use of Töpfer's reagent as an indicator in the titration of milk with hydrochloric acid. It was now thought desirable to determine whether the fat or the protein was responsible for this interference.

(a) Fresh skim milk was obtained which contained less than 0.02 per cent fat. On titration with hydrochloric acid, Töpfer's reagent functioned normally and changed at the proper pH. Thus it appears as though the casein is not the interfering factor.

(b) A sample of 20 per cent cream was acidified in the presence of an excess of Töpfer's reagent and centrifuged. The serum was pink, but the fat-

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protein layer had a yellow color. The casein was then removed by washing three times with N/10 sodium hydroxide, once with water, twice with N/10 hydrochloric acid, and once again with water. After this treatment the fat layer still retained the yellow color.

It appears probable that in this two-phase system the ionization of the Töpfer's reagent in the fat phase is negligible.

TITRATION OF SOYBEAN MEAL

Ten grams of soybean meal were suspended in 90 ml. of distilled water and titrated with hydrochloric acid in the presence of Töpfer's reagent.

During the titration with this acid, Töpfer's reagent remained yellow even after the pH was as low as 2.1 as determined potentiometrically. Again on the addition of 1 ml. of concentrated sulfuric acid, the reagent turned red. In this case, however, it was noted that if during the titration, 1 drop of the solution was removed and tested with Töpfer's reagent on a spot plate, the indicator turned red at the proper pH. This again indicated that some constituent of the soybean meal interferes with the use of Töpfer's reagent.

The pH range over which Töpfer's reagent changes color is wide, and thus even at best the use of the reagent is inaccurate. This fact, coupled with the interfering substances in milk, cream, soybean flour, and possibly other common foodstuffs, makes the use of Töpfer's reagent inadvisable in gastric analyses involving these foods. However, in routine gastric analysis, where the meal of bread and water is fed, it was found that Töpfer's reagent was satisfactory and agreed with potentiometric titration.

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A NOTE ON THE DECOMPOSITION OF NESSLER'S SOLUTION*

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RECENTLY we noticed that freshly prepared Nessler's solution on standing a short time in rubber-stoppered bottles formed a yellow precipitate. New solutions of Nessler's stock reagent and 10 per cent sodium hydroxide were prepared and mixed, and again cloudiness and precipitation occurred.

Another set of reagent bottles was obtained, and cork stoppers were used in place of rubber. No precipitation was observed with either the old or the new solutions. When rubber stoppers were substituted for cork, precipitation was noted. While trying to locate the cause of our trouble, we remembered that we had removed the bloom from rubber stoppers with acetone according to the method of Matviak.[1] The stoppers were not immersed in acetone but were wiped with a cloth moistened with acetone. They were washed thoroughly in water, dried and exposed to room air for a day or two prior to their use. No odor of acetone was detected from these stoppers.

The addition of a drop of acetone to Nessler's reagent caused immediate precipitation, and a like result was obtained when acetone vapor was blown over the mouth of a test tube containing Nessler's solution. Fumes arising from the use of acetone in the laboratory produced cloudiness, showing the extreme sensitivity of Nessler's reagent to acetone.

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DETERMINATION OF CALCIUM IN BILE*

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THE wet ashing method with nitric acid and perchloric acid, as described for grains by Giesiking, Snider, and Getz,[1] and for tissues by Buell,[2] may be applied to bile. It gives a water-soluble ash which is very satisfactory for the determination of calcium.

The procedure is as follows: Add to from 1.0 to 3.0 c.c. of hepatic bile in a 50 c.c. beaker twice the quantity of nitric acid and an equal amount of perchloric acid. For gall bladder bile use 0.5 to 1.0 c.c. samples and add four times the amount of nitric acid and twice the amount of perchloric acid. Heat to dryness at low heat on the electric hot plate. Take up the residue in water

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and transfer quantitatively to a 15 c.c. centrifuge tube, keeping the volume below 6 c.c. Add a drop of methyl red, enough ammonia to make the solution alkaline, and then 10 per cent acetic acid to approximately pH 4 to 5. Add 1 c.c. of ammonium oxalate (4 per cent) and proceed as in the Clark-Collip method for determination of calcium in blood.

Table I gives the results of some analyses.

TABLE I

BILE	AMOUNT TAKEN (C.C.)	POTASSIUM PERMANGANATE (C.C. 0.01N)		CALCIUM (MG./ SAMPLE)	CALCIUM FOUND (MG./ 100 C.C.)	CALCIUM THEORETICAL (MG./ 100 C.C.)	RECOVERY (%)
Human hepatic	2.0	0.40					
		0.38					
		0.36					
		0.37	0.38	0.076	3.8	Avg. 3.8	
	1.0 +	5.02					
	1 mg. Ca	5.04					
	(1.0 c.c.	5.07					
	solution)*	5.08	5.05	1.010	50.6	51.9	97.5
Dog hepatic	1.0	0.88					
		0.80	0.85	0.170	17.0		
	2.0	1.60					
		1.64	1.62	0.324	16.2		
	3.0	2.41					
		2.41	2.41	0.482	16.0	Avg. 16.4	
	3.0 +	4.66					
	0.5 mg. Ca	4.79	4.72	0.944	27.0	28.0	96.4
	1.0 + 1.0 mg. Ca	5.69		1.138	56.9	58.2	97.7
Dog gall bladder	0.5	0.90					
		0.91	0.91	0.182	36.4		
	1.0	1.77					
		1.84	1.80	0.360	36.0		
Dog hepatic	1.0	0.61					
		0.60	0.61	0.122	12.2		
	2.0	1.20					
		1.21	1.21	0.242	12.1		
	3.0	1.84					
		1.87	1.86	0.372	12.6	Avg. 12.4	
5 c.c. + 0.5 mg. Ca	1.0	0.97					
		1.01	0.99	0.198	19.8	20.3	97.5
5 c.c. + 1.0 mg. Ca	0.5	0.66					
		0.66	0.66	0.132	26.4	27.0	97.9
5 c.c. + 1.0 mg. Ca	1.0	1.24					
		1.25	1.25	0.250	25.0	27.0	92.6
Human hepatic	1.0	0.30					
		0.31	0.31	0.062	6.2		
	2.0	0.60					
		0.57	0.59	0.118	5.9		
	3.0	0.92					
		0.96	0.94	0.188	6.2	Avg. 6.1	
5 c.c. + 0.5 mg. Ca (0.5 c.c. solution)	1.0	0.77					
		0.78	0.78	0.156	15.6	14.7	106.1
5 c.c. + 1.0 mg. Ca (1.0 c.c. solution)	0.5	0.58					
		0.60	0.59	0.118	23.6	21.8	108.2
5 c.c. + 1.0 mg. Ca (1.0 c.c. solution)	1.0	1.11					
		1.10	1.11	0.222	22.2	21.8	101.8

*A solution containing 1 mg. of calcium per cubic centimeter was used in all instances where calcium was added to the bile.

Usually the residue obtained is white in color and dissolves readily in water. Occasionally a brown residue is obtained, which may be digested to whiteness by adding nitric acid and perchloric acid again. This is not necessary, however, as good results are obtainable even when the residue is slightly discolored. We have also found that if large amounts of organic material are present, as with gall bladder bile, the digestion mixture may turn dark brown or black when it reaches a small volume. If this happens, a second, or even a third, digestion with nitric acid and perchloric acid is necessary. In such instances care should be taken not to allow the dark mixture to evaporate to dryness since slight explosions may occur.

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FURTHER NOTES ON THE COLORIMETRIC DETERMINATION OF INULIN IN BLOOD AND URINE*

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WITH the development of a practical clinical method for measuring the glomerular filtration rate in man, clinical laboratories have been called upon to determine the concentration of inulin in blood and urine.[1] For this purpose a simple direct colorimetric method was described by Alving, Rubin, and Miller.[2] Certain difficulties which have been experienced by some laboratories in the routine application of this method may be overcome by the use of the following modifications and precautions.‡

Reagents.—Diphenylamine reagent. This reagent is now prepared as follows: To 250 c.c. of absolute ethyl alcohol in an Erlenmeyer flask 100 c.c. of C. P. concentrated hydrochloric acid are added. The mixture is then quickly cooled to room temperature. The change in volume resulting from the mixture of hydrochloric acid and alcohol is disregarded. Immediately before using the reagent, 20 c.c. of diphenylamine (from Merck or G. Frederick Smith Co., melting point 52.5° to 53.3° C.) in absolute alcohol are added. The alcohol-diphenylamine solution is made by adding 20 c.c. of absolute alcohol to 3.4 Gm. of diphenylamine in an Erlenmeyer flask. The final reagent is conveniently made from this solution and the acid-alcohol mixture by pouring them back and forth a few times from one Erlenmeyer flask to the other.

When prepared as described here, the reagent gives an appreciably deeper color in the reaction with inulin than the reagent employed previously. In

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‡Some precautions and modifications have already been described.[3, 4]

order to keep the blanks as low as possible we prefer to prepare the required quantity of this reagent fresh each day.

Removal of Fermentable Carbohydrate.—Commercial inulin solutions, which are satisfactory for intravenous clinical use, contain appreciable amounts of a non-inulin fermentable material. Since this substance gives additional blue color with diphenylamine, *it is essential that it be removed from both blood and urine samples by fermentation.* This procedure should be employed even when Benedict's qualitative test for reducing substances is negative on the urine.

After fermentation of the samples of plasma or urine, and removal of yeast cells by centrifugation, only the clear supernatant fluid is used for preparing protein-free filtrates.

A special, starch-free yeast can be obtained from the Fleischmann Yeast Co. It is necessary to wash this yeast as originally described.[2] It is worth while to use the special, washed yeast because with it the blank values are markedly reduced. For example, we obtain blank values on plasma below 0.5 mg. per 100 c.c. inulin equivalent.

Development of Color.—Reaction tubes. Improved, pressure-resistant tubes, manufactured by Kimble Glass Co., have the following specifications: "Special, round bottom, screw cap vials, 19 to 20 mm. outside diameter and 110 mm. long; made of N-51A No. 2 weight glass; one end threaded to fit an 18 mm. G.C.A. cap, other end round bottom. Vials must have standard fire-finished flat tops. Glass must be evenly distributed; vials must be lehred; lips must be square but not ground. Vials must have sandblasted spot as close to the shoulder as conveniently possible." New glass reaction tubes must be washed with soap and hot water before use in order to remove oily films from the glass. Then to remove traces of soap the tubes are rinsed thoroughly in hot water, filled with an acid-alcohol mixture similar to that described under "Reagents," stoppered, and heated in boiling water for at least fifteen minutes. After final rinsing with distilled water, these new tubes give satisfactory results. During subsequent use soap should not be employed; it is sufficient to clean the tubes by flushing with hot water, soaking in cleaning solution for at least one hour, and rinsing finally with distilled water.

Rubber liners. The use of replaceable, white rubber liners was recommended originally for the screw caps of the reaction tubes. The liners that we first obtained contained very little sulfur, which could easily be removed by boiling the liners in a dilute solution of sodium hydroxide. Liners must be free of sulfur which reacts with diphenylamine to produce a blue color similar to that given by inulin. When rubber liners are used, they must be cleaned in hot alcohol and rinsed in distilled water between determinations. If the glass reaction tubes or rubber liners are dirty, or are contaminated by traces of soap, the results will be less reproducible.

Plastic liners. It has become increasingly difficult to obtain durable rubber liners that are low in sulfur content. Because there is little prospect that this situation will improve, we have tried to use, with even more satisfactory results, 18-400 black phenol molded improved Lusterseal caps with glued-in Pliofilm pulp or *waxed* Vinylite pulp liners. The caps and liners do not have to be cleaned

before use. They must be discarded after being used once, but are cheap if purchased in quantity. Liners for this type of cap cannot yet be obtained separately. Satisfactory caps and liners are manufactured by Owens-Illinois Glass Co.*

Estimation of Color Intensity.—A No. 635 filter is somewhat more suitable for measuring light transmission than the No. 660 filter employed originally.†

Calibration.—Standard inulin solutions may be kept for months without deterioration if the inulin is dissolved in saturated benzoic acid. A standard, equivalent in color development to a 1 per cent solution of inulin, may be obtained from the United States Standard Products Co., Woodworth, Wis. This concern has also developed a satisfactory preparation of inulin for clinical use.

Determination of Blank Value of Reagents.—When several sets of analyses are performed, three blanks on water plus reagents, omitting yeast, or three tubes containing inulin of known concentration plus reagents, should be heated in each water bath with the solutions of unknown concentration in order to determine the "center-point setting" of the galvanometer.

The "center-point setting" may be determined by means of an inulin solution of known concentration as follows: With the known solution in the Evelyn colorimeter, the galvanometer is adjusted to correspond to the previously determined reading of the known solution on the calibration curve. The known solution is then taken out, and the galvanometer reading without any tube in place is taken as the "center-point setting."

The "center-point setting" can be determined a little more accurately for unknown solutions of high inulin concentration (low galvanometer reading) by the simultaneous analysis of a known solution containing 3.5 to 4.5 micrograms of inulin per cubic centimeter; but for unknowns of low concentration the use of blank analyses on water plus reagents is preferable, and for making the original calibration curve, it is imperative.

RESULTS

In analyzing inulin solutions of concentrations greater than 0.01 mg. per cubic centimeter, a small amount of blue precipitate is occasionally formed in the colorimeter tube. Ordinarily the error introduced by this precipitate is small, but the formation of the precipitate can be prevented by the proper dilution of samples before analysis. Whenever possible the unknown solution should be diluted to give a reading between 30 and 70 on the galvanometer scale of the Evelyn photoelectric colorimeter.

It must be emphasized that for most reproducible results the time of boiling should be standardized to exactly sixty minutes. The reaction is stopped by cooling the tubes immediately to room temperature. The colorimetric determinations should be carried out approximately thirty minutes after the tubes are removed from the boiling water bath.

*Reaction tubes, caps, and liners may be obtained from regular laboratory supply houses. We obtain our supply through E. H. Sargent and Co., Chicago, Ill.

†Filter No. 635, supplied by Rubicon Co., Philadelphia, Pa., permits transmission of approximately 95 per cent of the light between 618 and 680 millimicrons, the approximate mean wave length of the transmitted band being 635 millimicrons.

In performing inulin clearances, the effect of analytical errors on the clearance values can be reduced to a minimum by heating corresponding plasma and urine samples together in the water bath, instead of analyzing plasma and urine samples at different times.

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A CHEAPER NESSLER'S REAGENT BY THE USE OF MERCURIC OXIDE*

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INTRODUCTION

With the general rise in the price of chemicals at the present time, any economy in preparing large batches of Nessler's reagent should be of interest to most laboratories. Formation of the desired double halide from the *oxide* of mercury (and twice the usual equivalent of potassium iodide) is cheaper than its preparation from the very expensive mercuric iodide, or even from elementary iodine and mercury.

Nessler's reagent, as we all know, is essentially a strongly alkaline solution of a certain double iodide of potassium and mercury. Reaction with ammonia yields a colloidal[1] compound which in dilute solutions is evident as a yellow to brown color, and in less dilute ones (where the concentration of ammonia exceeds a few milligrams per hundred cubic centimeters) as a precipitate.

There is only slight doubt as to the composition of the effective double iodide in this old[2] and much-modified reagent. It is generally agreed to be formed from the equivalent of two moles of potassium iodide to each one of mercuric iodide: $2KI \cdot HgI_2$ or K_2HgI_4. Friend[3] leaves the matter uncertain, but Treadwell and Hall[4] give the above salt in an equation for nesslerization. Mellor,[5] in his familiar *Comprehensive Treatise*, submits the usual mass of conflicting statements, but later (in his one-volume edition[6]) he also presents K_2HgI_4 in an equation and states that the effective ratio of mercury to iodine should be 1:2.54, which corresponds exactly to the above compound. Yoe[7] likewise agrees, as do Latimer and Hildebrand.[8]

*From the Research Department of the Barnard Free Skin and Cancer Hospital, St. Louis. Received for publication, July 14, 1941.

The double iodide evidently dissociates scarcely at all to give mercuric ions, for the addition of hydroxyl ions in considerable concentration may produce no precipitate of mercuric oxide. Ionization must largely occur thus:

$$K_2HgI_4 \rightarrow 2K^+ + HgI^=_4.$$

In the presence of ammonium ion or ammonia as well as of alkali, this complex ion reacts to yield a peculiar, highly colored compound, probably a polymer. Equations such as these are given:

(1) $4K^+ + 2HgI^=_4 + 4OH^- + NH^+_4 \rightarrow NHg_2I \cdot H_2O + 3H_2O + 4K^+ + 7I^-$.[4]
(2) $2K_2HgI_4 + 3KOH + NH_3 \rightarrow HgOH \cdot NH \cdot HgI + 7KI + 2H_2O$.[5]

The chromatic substance, "mercuric hydroxiodamide"[5] or "oxydimercuric ammonium iodide,"[6] is of very doubtful composition. It is said[4] to be the iodide of "Millon's base," the white product obtained when aqua ammonia acts upon mercuric oxide. There is a host of formulas given for Millon's base and still more for Nessler's precipitate. Many texts are agreed that the latter compound has an *empirical* formula of NH_2Hg_2OI,[9] which is variously written as: $OH \cdot Hg \cdot NH \cdot HgI$,[3] $NHg_2I \cdot H_2O$,[4, 8, 22] $NH_2 \cdot Hg \cdot OHgI$,[10] $HgOH \cdot NH \cdot HgI$,[6] $HgO \cdot Hg(NH_2)I$,[7] etc. Franklin[11] in 1907 also gave Hg_2NH_2OI, but later[12] changed this to either $2HgO \cdot NH_3 \cdot H_2O$ or $4HgO \cdot 2NH_3 \cdot H_2O$. The most recent attempts at analyzing this baffling compound were by Nichols and Willits,[1] who claim that it is $NH_2 \cdot Hg_2I_3$, and give this expression for nesslerization:—

(1) $2(HgI_2 \cdot 2KI) + 2NH_3 \rightarrow 2(NH_3 \cdot HgI_2) + 2KI$.
(2) $2(NH_3 \cdot HgI_2) \rightarrow NH_2Hg_2I_3 + NH_4I$.
Or, combining: $2(HgI_2 \cdot 2KI) + 2NH_3 \rightarrow NH_2Hg_2I_3 + 2KI + NH_4I$.

The necessity for alkali is not obvious in their equations. The colored substance, the authors further state, is highly insoluble and forms an electronegative suspensoid. This lyophobic nature is certainly indicated by the great gain in stability when soluble gums[13] are previously added to nesslerized solutions.

If we assume then that the effective double iodide is $2KI \cdot HgI_2$, and calculate the ratio of potassium iodide to mercuric iodide for various Nessler's reagents, we see that in the more satisfactory preparations this ratio, by design or by chance, closely approaches our theoretical ratio or slightly exceeds it for the sake of stability. $2KI : HgI_2 = (2 \times 166.0) : 455.4$ = approximately 332/455, or about 0.73.

REAGENT	REFERENCE	% KI	% HgI_2	RATIO	% NaOH
Frerichs and Mannheim	14	2.5	3.5	0.71	15 (KOH)
Folin and Denis	15	2.25	3.0	0.75	2.0
Folin-Wu	16	1.125	1.50	0.75	7.0
Bock-Benedict	17	7.0	10.0	0.70	10.0
Koch-McMeekin	18	1.02	1.38	0.74	8.3
Vanselow	19	3.49	4.55	0.77	11.2 (KOH)

In fact, if one deviates very far from this figure, the sensitivity of the reagent decreases enormously. For example, when employing equal molarities of the potassium and mercuric iodides, a color will not develop with ammonia even when the reagent is strongly alkaline. It *is* desirable that there be just sufficient excess of potassium iodide to keep the mercuric iodide in solu-

tion; but little more or sensitivity will suffer. Vanselow[19] recommends a 5 per cent excess of potassium iodide over that required to form the hypothetical K_2HgI_4, and thus the ratio in his reagent is 0.77. Other factors also reduce the sensitivity. With insufficient free hydroxyl ions, no color will develop at all, but great excess of alkali will produce a precipitate, probably mercuric oxide. Certain foreign salts, such as bicarbonates[4] or chlorides,[18] lower the intensity of the Nessler color. Temperature is also stated[20] to be rather influential.

The oldest method of preparing Nessler's reagent is the somewhat arbitrary one of adding an excess of potassium iodide to a saturated solution of mercuric chloride[21] (and then considerable base).

$$HgCl_2 + 4KI \longrightarrow K_2HgI_4 + 2KCl.$$

This technique is still given in many texts,[9, 22] but it is best suited for water analysis and such instances where the concentration of ammonia is quite low. Otherwise, the potassium chloride adds unwanted electrolyte which increases the tendency of the suspensoid to precipitate.

The time-honored procedure[15, 16, 18] of reacting metallic mercury with a triiodide solution yields the compound desired, but it is inconvenient and it appears that it is becoming obsolete.

$$2KI{\cdot}I_2 + Hg \longrightarrow K_2HgI_4.$$

When mercuric iodide, free of such impurities as the sulfide and mercurous compounds, became readily available, the modification of dissolving mercuric iodide in strong, hot potassium iodide solution was adopted.[14, 17]

$$2KI + HgI_2 \longrightarrow K_2HgI_4.$$

Dawson,[23] in his equilibrium researches, found evidence that in solutions where the dissolved mercuric iodide is relatively low, the chief complex electrolyte is K_2HgI_4.

The proposed method is this:

$$HgO + 4KI + H_2O \longrightarrow K_2HgI_4 + 2KOH.$$

Here we have two end products, but both are needed for the reagent (the amount of alkali formed supplies only a fraction of that required). Rupp and Schirmer cited by Mellor[5] prepared the double iodide by the last reaction and proposed it as a method for standard alkali.

Friend,[3] and Latimer and Hildebrand,[8] also give the above equation as a reaction of mercuric oxide. This reaction was once used[24, 25] as a technique for providing standard alkali from a known weight of the pure oxide. Strangely, after a rather thorough search of the literature, I have encountered no mention of the utilization of this reaction of mercuric oxide to prepare Nessler's reagent.

PROCEDURE

Directions are given for the substitution of mercuric oxide in the preparation of the Koch-McMeekin[18] solution only, as this modification of the old Folin-Wu formula is probably the best Nessler's reagent as regards sensitivity, stability, and economy of materials that is in general clinical use. I have

prepared this formula (and others) by the authors' original method, with mercuric iodide, and with the oxide, and have found the three solutions apparently identical in excellence and composition.

Dissolve 19.1 Gm. of A.R. (analytical reagent quality) red mercuric oxide powder in a solution of 60 Gm. of A.R. potassium iodide in about 100 c.c. of ammonia-free water. Heat on an electric hot plate and stir until clear. The last bit of the solid dissolves with difficulty. Cool the deep yellow solution of the double iodide, make to 200 c.c., and then stir into 975 c.c. of pure 10 per cent sodium hydroxide. This 1,175 c.c. volume of the original Koch-McMeekin reagent can be rather inconvenient. A liter could be prepared by using somewhat less of the iodide stock solution, thus: pour 160 c.c. of 50 per cent sodium hydroxide solution into about 600 c.c. of ammonia-free water. Add only 170 c.c. of the above iodide stock (with stirring) and dilute all to a liter with redistilled water. The objection may be raised that some of the iodide stock is left over. To avoid this, we can use a stock solution somewhat less concentrated than the original:

Dissolve 16.2 Gm. of A.R. red mercuric oxide in a solution of 51 Gm. of A.R. potassium iodide in 100 to 150 c.c. of ammonia-free water. Heat on an electric hot plate and add the powder in small portions, stirring until all is dissolved. Then dilute the double iodide solution thus obtained with about an equal volume of redistilled water and let cool. Dilute also 160 c.c. of clear (centrifuge if necessary) 50 per cent sodium hydroxide solution with about one-half liter of pure water. Pour in the iodide stock and make to one liter.

Red mercuric oxide is recommended since it costs somewhat less than the yellow form. The latter differs only in being of smaller particle size[8] and should be quite as satisfactory chemically, and perhaps more readily soluble.

How familiar is the phrase, "let stand to clear," but if the Nessler's reagent is prepared of pure materials, it is almost perfectly free of turbidity at once. Much purchased distilled water, especially that from raw river water, contains a surprising amount of ammonia and organic matter. If this is so, redistil that used for the reagent from an acid-permanganate solution. (In the interest of safety, add only enough potassium permanganate to maintain a decided purple color and just a small percentage of sulfuric acid.) Carbonate-free sodium hydroxide will be assured if one uses the convenient 50 per cent solution.

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MEDICAL ILLUSTRATION

THE APPLICATION AND WEARING OF FACIAL PROSTHESES*

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NUMEROUS articles have appeared in the medical and dental literature with regard to the manufacture of moulage prostheses or artificial parts to be worn on the human face and body. The most outstanding of these are by Kazanjian, Rowe, and Young,[1] Bercowitsch,[2] Zinsser,[3] Clarke,[4, 5] and Bulbulian.[6, 7] The majority of the prostheses considered in these articles were artificial noses, ears, cheeks, and eyes in which parts of the orbits and lids have been reconstructed rather than false teeth and gums that fit adjacent to mucous membrane. The prostheses to be discussed by me fit primarily next to skin surfaces and are held in place by mechanical devices, such as eyeglass frames, or an adhesive, such as gum mastic, dissolved in the proper solvent. The material already published did not explain in detail the actual wearing of prostheses. Instead, the manufacture of the prosthesis was considered, and methods were given for attachment by mechanical devices or by an adhesive. These articles left the prosthetist and his patient to determine by experimentation and experience the most comfortable adhesive mixture to use and the length of time required to rest the tissues. For example, some wearers of artificial parts on the face prefer to wear them continuously for a few days, others prefer to remove them every night. This depends largely on the extent of the restoration and the materials used.

I have been associated with two physicians who have had prostheses made months ago and are wearing them at the present time. Since these men themselves have facial restorations, they are exceptionally well qualified to know from firsthand experience the problems that arise during the application and wearing of prostheses. Furthermore, as physicians they understand the nature of any irritations that may begin and know how to correct them. One of these cases represents a partially restored nose that was required after the removal of an epithelioma; the other is a partially restored ear designed to correct a defect resulting from an accident. I am, therefore, indebted to these two men, who prefer to remain anonymous, for the following information and description of their actual experiences during the wearing of their respective prostheses.

PRECAUTIONS TO BE TAKEN IN MANUFACTURE OF PROSTHESES

In the manufacture of a prosthesis it is essential to use the utmost care in the preparation of both surfaces of the material, that is, the outer or visible surface and the surface that will come in contact with the skin. The exposed

*From the Department of Art, University of Maryland School of Medicine.

contours should be regular, smooth, and lifelike, with the correct proportions and color. The contact surface should be smooth, without sharp points or granular areas that do not make complete contact. It must fit snugly and completely to the skin on which it will be attached; any irregularity or depression in the skin must be filled with a corresponding negative impression on the moulage. It is for this reason that flexible materials, such as latex and gelatin-glycerin-sorbital mixtures are more practical than the hard, firm, unyielding substances. Metal, vulcanite, and the synthetic resins, such as vernonite and vinyl acetate, belong to the latter group.

Surface area should not be sacrificed for cosmetic effect in preparing the contacting surface. In other words, the surface area should be sufficiently extensive to approximate a considerable portion of the skin area when applied to it with the adhesive substance; otherwise the border of the prosthesis will not approximate closely the dermal structure. The edges will be more apparent and fixation will be unsatisfactory. This statement from the wearer of a prosthesis came as a surprise, because I labored under the assumption that the smaller the skin surface covered by an artificial part, the more realistic the effect. In his opinion sufficient skin surface must be covered to make the artificial part feel comfortable and at the same time give complete assurance that it will not fall off. Naturally, this matter should be tempered according to the size and weight of the prosthesis and the strength of the adhesive used. When making a prosthesis, the seam or junction between the natural and artificial skin surfaces should come at points, lines, or areas that will prevent detection as far as possible. Folds and character lines within the skin surface offer the best areas for forming the joining lines. When such details are considered, the moulage maker may take advantage of them in making the prosthesis to produce surfaces that will support the weight of the prosthesis and at the same time escape detection.

ADHESIVE MATERIALS

Bercowitsch[2] and Zinsser[3] advised the use of gum mastic dissolved in alcohol as an adhesive to hold a nose or an ear prosthesis to the skin surface. In trying this mixture I found that the alcohol dried too slowly from the mastic to effect satisfactory adhesion. Furthermore, the mastic turned milky in the presence of moisture, an effect known as *bloom* in the field of art. Since gum dammar does not react in this manner, I substituted it for the mastic.

Gum dammar and gum mastic possess the following qualities: (1) They are transparent when in solution. (2) They are insoluble in water. (3) They are non-irritating. However, I have heard of one case in which the gum mastic solution was blamed for causing a skin rash at the place where the prosthesis joined the skin. I am inclined to believe that this was the result of an idiosyncrasy to the gum, or that there was a mechanical irritation created by removing the part too frequently. In numerous other instances the gum mastic was used properly over a period of years without any detrimental effect. (4) An excess of either gum dammar or gum mastic or any quantity of these which remains dried on the skin or the prosthesis may be removed with a 70 per cent solution of alcohol. (5) These ingredients are inexpensive, a year's supply costing only a few cents.

(6) Both gum mastic and gum dammar are not affected by normal temperature changes. Gum dammar is not affected by excess humidity, and gum mastic is not likely to be changed to any great extent. The wearer may notice that the gum mastic will bloom when it is applied in rainy weather. However, this is of little consequence. (7) A normal prosthesis may be attached by the use of these materials in a few minutes, fifteen minutes at the most.

To overcome the slow drying of the alcohol, as recommended by Bercowitsch and Zinsser, I used a mixture of equal parts of alcohol and ether in which the gum dammar was dissolved. The patient was advised to paint the gum dammar solution on the surface of the prosthesis that was to come into contact with the skin surface, then put the prosthesis in place and hold it for a few minutes until complete and comfortable adhesion was effected. This required only a few minutes. I instructed the patient to increase the amount of ether in the solution if he wanted quicker adhesion, and to add more alcohol if the adhesion took place too quickly or dried before the prosthesis could be put in place.

This reasoning seemed logical to me, but I found that one wearer preferred to use ether entirely as a solvent for gum mastic. The fact that this gave satisfactory results over a period of years speaks for itself. The wearer claims that the gum mastic is more effective when dissolved in ether, and if kept in solution will prove a more uniform and adequate adhesive agent than if prepared for each individual application. It is best applied to the prosthesis by means of a medicine dropper, and the density of the solution should be such that it can be drawn into the dropper with ease. The ether evaporates very readily from the solution if the container is left open for any length of time and should be replaced from time to time if the mixture becomes too thick. In the event the solution becomes too thin, additional gum should be added. I always prepare a bottle of the gum solution and give it to the patient with his prosthesis. Instead of the medicine dropper, I use a bottle with a stopper that contains a small bristle brush. This brush is used to apply the adhesive to the contacting surfaces. After the adhesive is applied, the prosthesis is put in place and held with firm, constant, and regular pressure until the solvent evaporates from the gum and complete adhesion takes place.

WEARING OF THE PROSTHESIS

The prosthesis, after its application, can usually be worn for about three days. The gum mastic then becomes only loosely adherent and removal at that stage is simple, only a small amount of traction being necessary. If the prosthesis is removed at such time there will be no skin abrasions or irritations. The prosthesis may then be cleaned with alcohol and replaced. There is no reason why a prosthesis made of latex or a gelatin-glycerin composition should not be worn at night. As a matter of fact, it is unwise to detach it too frequently, particularly when it is attached firmly to the skin. Of course, the size of the prosthesis and the material from which it is made may have some bearing on these instructions. If the prosthesis is attached to the eyeglass frame it may be possible to dispense with an adhesive altogether. This depends upon the location and extent of the lesion and the size of the prosthesis.

CARE OF THE SKIN

When the prosthesis is removed, the dried nonadhesive debris of the gum-solvent solution should be cleaned away carefully with a 70 per cent alcohol solution. Gentle massage with a soft cloth dampened with alcohol is all that is necessary. The generous application of alcohol to the surrounding skin surface with the fingers will be found comforting and helpful. It will also be found practical and wise to leave off the prosthesis occasionally for several hours, as during sleep. The application of tincture of green soap before this replacement is made may aid in protecting the surface.

In a recent communication one of these physicians supplied the following information: "I have been using the moulage prosthesis for two years and during this period have had absolutely no skin or other reaction of any sort in spite of the fact that my skin is unusually sensitive. My prosthesis is most satisfactory and is not noticed by the public; however, upon a few occasions some of my medical friends have asked me who did the plastic operation and were wondering from what area the skin was removed. It has been my choice to remove it each night, replacing it in the morning, which is done in less than a minute's time."

If the principles here outlined are followed carefully, a satisfactory wearing of the prosthesis will be experienced with the least amount of physical and mental discomfort and the maximum in cosmetic efficiency.

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Erratum

On page 1799 of the August number of the Journal in the article by Theodore S. Rosen, M.D., and George W. Lubinsky, A.B., entitled "Freezing of Tissues With 'Dry Ice' for Microtome Sectioning of the Entire Brain," the first two sentences in the paragraph under "Freezing Apparatus" should read: "The freezing attachment consists essentially of (1) a metal plate upon which the slab of tissue is frozen, and (2) a means of keeping the 'ice' *in firm contact with the metal plate. The plate is cooled by the 'ice'* and in turn chills the tissue, at the same time anchoring the tissue to the plate." The words that are italicized were omitted from the text.

DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

PNEUMOCOCCI, Sensitivity of to Sulfapyridine, A Rapid Qualitative Test for Resistance, Moore, F. J., Thomas, R. E., and Hoyt, A. J. A. M. A. **117:** 437, 1941.

Each of 4 mice was inoculated intraperitoneally with 0.2 c.c. of 10 per cent sulfapyridine suspended in 20 per cent acacia. Sputum (or pus) was made grossly homogeneous and liquid by mixing with a minimum necessary amount of sterile saline solution. Within the first two hours after treatment, 4 treated and, in addition, 3 untreated mice were alternately inoculated with the sputum mixture, as large a volume as possible (up to 1 c.c.) being used for each mouse. At various intervals thereafter peritoneal exudate was removed from each mouse and examined rapidly to determine the number and the morphology of the pneumococci. Efforts to make accurate counts were avoided. The test was read when growth of pneumococci in the untreated mice had become unquestionable (as indicated by hundreds per oil-immersion field), and at no earlier time. If, at this time, the treated mice showed no increase in the number of pneumococci, or if there had been scanty growth with a preponderance of atypical forms, the strain was called sensitive; if smears of treated and control mice were virtually indistinguishable, the strain was called resistant (fast or refractory).

TRICHINOSIS, Wyrens, R. G., Tillisch, J. H., and Magath, T. B. J. A. M. A. **117:** 428, 1941.

In an analysis of 19 cases of clinical trichinosis and 21 cases of asymptomatic infection incidental diagnoses were made during routine examination of surgical pathologic material. Included in the series are 2 cases suggestive of repeated infection.

Asymptomatic infection with *T. spiralis* and clinical trichinosis are two conditions which should be clearly separated.

SPOTTED FEVER, Rocky Mountain, Hutton, J. G. J. A. M. A. **117:** 413, 1941.

The literature shows that cases of Rocky Mountain spotted fever have been reported from thirty-seven of the forty-eight states.

Rocky Mountain spotted fever is transmitted to human beings through infected ticks, and the causative organism is *Dermacentroxenus rickettsii*.

Three types of the disease are recognized: the mild, the severe, and the fulminating.

The cutaneous manifestations of Rocky Mountain spotted fever are characteristic.

A drug exerting any great amount of specific action against this infection has not been found.

Vaccine gives partial protection for a period of one year or less.

The United States Public Health Service vaccine for Rocky Mountain spotted fever is prepared at the Rocky Mountain Laboratory of the National Institute of Health at Hamilton, Mont.

The vaccine is given in two doses of 2 c.c. each at an interval of five days, either subcutaneously or intramuscularly. The known data indicate that the vaccine fully protects the majority of persons against the less virulent strains but that the average person is only partially protected against the highly fatal type of Rocky Mountain spotted fever, and that the maximum degree of protection conferred is retained for less than one year.

GONOCOCCIC INFECTIONS, Cutaneous Diagnosis of, Corbus, B. C., and Corbus, B. C., Jr. J. A. M. A. **116**: 113, 1941.

A person infected with a specific organism is in a state of hypersensitivity to that organism as long as it remains within the body.

In a control series of 100 persons known not to be infected with gonococci, 85 per cent gave negative reactions to the cutaneous test described.

According to the authors' experience, this test as a diagnostic adjunct in gonococcic infections is more valuable than any other procedure previously available.

The antigen consists of 0.1 c.c. of a standard bouillon culture filtrate with 0.1 c.c. of bouillon as a control.

COLOR INDEX, A Simple Hemoglobin-Red Cell Ratio to Replace the, Isaacs, R. J. A. M. A. **116**: 2258, 1941.

A simple relationship between the hemoglobin content of the blood and the number of red blood cells may be expressed by this formula for "normal": Three times the number of grams of hemoglobin per hundred cubic centimeters divided by the number of hundreds of thousands of red blood cells per cubic millimeter equals 1. It is distinctly less than 1 in hypochromic anemias and more than 1 in hyperchromic conditions.

URINE, Method for Rapid Alkalinization of, Bick, M., and Drevermann, E. B. M. J. Australia **1**: 223, 1941.

The authors advocate the immediate intravenous administration of 20 c.c. of the sodium lactate-sodium bicarbonate solution on the first appearance of any symptoms suggestive of the occurrence of hemolysis after blood transfusion. This is, however, only the immediate treatment, and as large quantities of fluid are required to maintain the diuresis, the purpose of the injection is to alkalinize the urine rapidly in order to permit time for the setting up of a continuous intravenous infusion of isotonic sodium lactate solution.

HEMOPHILIA, Diagnosis of, Quick, A. J. Am. J. M. Sc. **201**: 469, 1941.

The blood of a true case of hemophilia should show the following:

1. Coagulation time (Lee White test), over eight minutes at 37.5° C.
2. Coagulation time of recalcified plasma:
   a. High speed centrifugation, over five minutes.
   b. High speed centrifugation, over three minutes.
   c. Clotting time should decrease on standing.
3. Prothrombin concentration (Quick's method), above 70 per cent.
4. Bleeding time (Duke's method), not over four minutes.
5. Clot retraction, not over sixty minutes after coagulation occurs.
6. Tourniquet test (Rumpel-Leede technique), not over four petechia in specified area.

BLOOD DONORS, Response of, to Iron, Santy, A. C. Am. J. M. Sc. **200**: 790, 1941.

The hemoglobin response of 27 active professional blood donors was studied under iron therapy and without medication.

It was found that hemoglobin regeneration after donations took place eight times more rapidly when iron was given.

The return to the previous level was complete in an average of eleven days after a 500 c.c. donation under iron therapy, whereas a far longer period was necessary when iron was withheld.

A rough calculation of the amount of hemoglobin in the total circulation revealed the fact that the average active donor in this series could not maintain a positive balance without iron medication.

It was found that the response to iron usually continued after its withdrawal, indicating that the most effective time to administer iron is before blood loss.

It is concluded that the diet of regular donors should be supplemented with iron. Ferrous sulfate, because of its convenience and freedom from reactions, was regarded as a very satisfactory form of iron for blood donors.

"STORED" BLOOD. Effect of Glucose in the Preservation of Citrated Human Blood Stored at 4-6° C., Belk, W. P., and Rosenstein, F. Am. J. M. Sc. 200: 841, 1941.

Glucose (5.4 per cent) prevents the hemolysis of erythrocytes of whole citrated human blood kept at 4° to 6° C. for three to four weeks. With citrate alone hemolysis appears near the end of the first week.

This preserving effect is less marked on granulocytes, platelets, and on the clot-forming properties.

One part of isotonic glucose to two parts of blood is effective, and has the advantage of increasing not too greatly the amount of fluid to be injected.

It must not be thought that blood preserved by chilling and the addition of glucose remains in a state of hibernation, or suspended animation. The processes of decay go on steadily, and are retarded only by the favorable environment. There is, therefore, a strict limit to the time that blood for transfusion may properly be stored. This limit is shorter than is generally supposed due to the fact that these red blood cells become incapable of substituting for the cells of the recipient prior to suffering any hemolysis.

"STORED" BLOOD: Survival Time of Erythrocytes of Citrated Human Blood Stored at 4-6° C., Belk, W. P., and Barnes, B. C. Am. J. M. Sc. 200: 838, 1941.

The erythrocytes of citrated human blood kept at 4° to 6° C. were found to survive in recipients as long as the cells of fresh blood when storage time did not exceed two or three days. After this they disappeared with increasing rapidity as storage time increased, until, after seven or more days in the "bank" their post transfusion survival was in no case longer than twenty-four or forty-eight hours.

GONOCOCCUS, Preservation of, in Frozen Urines and Broth, Wortman, M. S., Gronau, A., Deakin, R., and Love, F. Ven. Dis. Inform. 22: 195, 1941.

Routine Method:

The urethral discharge of male patients with gonorrhea was collected on a sterile swab which was then immersed in a serologic tube containing 1.5 c.c. of infusion broth. Subsequently the specimen was plated out on a chocolate agar plate. If there was no discharge, the first portion of the urine was collected in a sterile centrifuge tube. The sediment was taken up on a swab after centrifugation and transferred into a broth tube. A culture was then made as stated above.

In female patients the cervix was exposed by means of a speculum, avoiding the use of lubricants. A specimen was taken from the urethra and cervix with a swab which was then immersed in a broth tube and later plated out as described.

Freezing Method:

(A) Broths. A routine culture was made from the discharge in both male and female patients, and the swab was then placed back into the broth tube. The broth tube was immersed in the freezing mixture of dry ice and alcohol (temperature about −72° C.). Freezing occurred in about fifteen to twenty seconds. The specimens were transferred when frozen to a thermos bottle containing dry ice.

(B) Urines. Urines collected in sterile 15 c.c. centrifuge tubes were frozen by immersing the tubes in the freezing mixture. Freezing occurred in about one and one-half to two minutes. The tubes were transferred after freezing to a thermos bottle containing dry ice.

TUBERCLE BACILLI, Demonstration of, Sasano, K. T., Caldwell, D. E., Needham, E. L., and Medlar, E. M. Am. Rev. Tuberc. 43: 263, 1941.

A comparison of results obtained from culturing and inoculating guinea pigs for the detection of tubercle bacilli in 1,270 specimens from human sources is presented. The results obtained from culturing the spleens from 100 consecutive tuberculous guinea pigs, using the technique briefly outlined in the text, are given. The advantages and disadvantages of each method are discussed. To substitute the cultural method for the guinea pig inoculation procedure seems inadvisable. The utilization of both methods counterbalances certain inherent disadvantages in each. As a routine procedure a combination of the two methods will make possible a more accurate detection of tubercle bacilli present in small numbers than is possible where only one or the other method is used.

TUBERCLE BACILLI, Culture of, Steenken, W., Jr., and Smith, M. M. Am. Rev. Tuberc. 43: 309, 1941.

A fifty-hour sputum specimen is collected in a 100 c.c. wide-mouthed bottle, stoppered with a good grade of cork. An equal volume of 4 per cent sodium hydroxide is added, and the mixture is homogenized for ten minutes in a shaking machine, followed by one hour's incubation at 37.5° C. It is then centrifuged for ten minutes at high speed in sterile pyrex tubes which have been sealed with sterile rubber caps (type used for capping babies' milk bottles). The sediment is neutralized with normal hydrochloric acid containing 10 per cent by volume of phenol red indicator, and adjusted on the acid side to a pH of about 6.4 (it is best to approach the final pH with either N/10 hydrochloric acid or N/10 sodium hydroxide as may be required).

This suspended sediment is divided into three equal portions. Two of these portions are transferred to two small vials with round bottoms that will stand entrifuging and which can be stoppered with small corks. (Small round bottom 5 c.c. vaccine vials are well suited for this purpose.) To one vial, 5 c.c. of a 1:1,000 solution of malachite green is added, and to the other vial, 5 c.c. of a 1:10,000 solution. The third vial is kept as a control. The vials which contain the dye are then stoppered and agitated in the shaking machine for ten minutes, then centrifuged for ten minutes at high speed. The supernatant fluid is pipetted off with a sterile capillary pipette. The sediment is then suspended in 1 c.c. of sterile distilled water.

STREPTOCOCCI, Comparison of Beta-Hemolytic on Maltose-Blood-Agar. A Presumptive Test for Group A, Simmons, R. T., and Wilson, H. M. J. Australia 1: 74, 1941.

A total of 869 strains of beta hemolytic streptococci of human origin of groups A, B, C, and G were examined on maltose-blood-agar plates as a presumptive means of distinguishing strains of group A from the other three groups, which are commonly found in man but are rarely pathogenic to man.

Of 500 strains of group A streptococci, all but four (0.8 per cent) were recognized. Of 369 strains of groups B, C, and G, all but 15 (4 per cent) were recognized as not of group A.

As a presumptive test for group A streptococci, the medium maltose-blood-agar was useful, and, with few exceptions, reliable. As a presumptive test for group B among hemolytic streptococci of human origin, it was quite reliable. Groups C and G were not distinguishable from one another, but most strains of groups C and G were distinguishable from group A, and all strains were distinguishable from group B.